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Cellular Vesicle Called "Exosome," Preparation and Use Thereof in Immune Stimulation

[Vésicule cellulaire dénommée "exosome," leur préparation et utilisation dans la stimulation d'une réponse immunitaire]

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The object of the invention is a novel process for sensitizing antigen-presenting cells, novel means for implementing the process, and novel membrane vesicles with immunogenic properties.

Since demonstration of the existence of specific cytotoxic CD8+ T lymphocytes of tumor antigens presented in the context of Class 1 molecules (Rosenberg et al., 1996; Boon, 1992), several laboratories have successfully shown that anti-tumor immunotherapy is an effective therapeutic strategy in animal models (Pardoll, 1995). The principle behind immunotherapy is to induce an effective immune response against specific tumor antigens. At the present time, this has been performed in various ways. First of all, tumor cells that express recombinant co-stimulation molecules and/or immunomodulative cytokines are able to stimulate anti-tumor responses capable of eradicating solid tumors in vivo (Zitvogel et al., 1996 [a]). Likewise, peptides derived from tumor antigens (or from exogenous antigens expressed in the tumor cells) that are injected in various chemical forms, including the use of liposomes or viruses (adenovirus or poxvirus, for example) as vectors, are able to cause regression of the tumors. Finally, professional antigen presenting cells, like dendritic cells sensitized with peptides derived from tumor antigens, that are reinjected in vivo induce powerful anti-tumor responses, as well as regression of established solid tumors, in mice (Mayordomo et al., 1995).

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[1] Numbers in the margin indicate pagination in the foreign text.

Immunotherapy based on the use of dendritic cells has proven its efficacy in studies performed on mice. Given this fact, this therapy was recently transposed into clinical practice. In the U.S., trials are currently underway in order to demonstrate that dendritic cells loaded with tumor peptides significantly increase the frequency of specific cytotoxic T cells (CTL).

An initial limitation to this approach is the sensitization of dendritic cells with tumor-antigen-derived peptides. Indeed, in most tumors, specific antigens have not been identified. Specific antigens of the tumor are only known in cases of virus-induced tumors (cervical carcinoma), in melanoma (self antigens, mutated antigens, differentiation antigens), or in a small percentage of breast tumors (oncogens, or products of tumor-suppressing genes that have undergone mutations). However, the direct involvement of these peptides or tumor antigens in the elimination of tumors in humans remains to be demonstrated. New methods for sensitizing antigen presenting cells such as dendritic cells are therefore necessary. The goal of these methods is to induce specific anti-tumor responses in the context of MHC Class I and Class II molecules.

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Most currently-used methods for sensitizing dendritic cells use peptides corresponding to epitopes presented in association with Class I molecules and identified in the tumor cells thanks to clones of specific CTLs of the tumor. However, these methods are not optimal because they do not take into account the epitopes recognized in the context of Class II molecules that are

critical for the proliferation of auxiliary T lymphocytes necessary for obtaining optimal cytotoxic responses. Moreover, epitopes presented by tumor cells and those presented by antigen presenting cells (such as dendritic cells, for example) are probably not identical. Finally, tumor peptides recognized by CTLs are only available for a small percentage of patients having Class I molecules with the appropriate haplotype.

The ideal sensitization method, one that could be applied to any tumor with a minimal risk of immunoselection, should not be limited to a small number of identified tumor antigens. Likewise, such a method should use intact protein antigens rather than peptides, in order to enable the dendritic cell to prepare them and to present the adequate combination of peptides in association with the Class I and Class II molecules, and should be applicable to all individuals.

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Recently, Gilboa and colleagues (Boczkowsky et al., 1996) showed that messenger RNA prepared from tumor biopsies loaded into the dendritic cells may have an anti-tumor effect in vivo. However, the RNA are very unstable and the potentially involved quantity of RNA compared to total RNA is very low. Zitvogel et al., (Zitvogel et al., 1996 [b]) have shown that tumor peptides prepared from an acid tumor eluate (peptide acid eluate: PAE) may be used for loading dendritic cells. These cells, thus loaded, are able to cause regression in tumors once injected. However, in the case of tumors that do not express Class I molecules (which represent the majority of metastatic tumors in humans), or in the

case of tumors that cannot be dissociated in a cellular suspension, the approach using acid eluates is not very effective and is not reproducible.

Another limitation to immunotherapy based on using dendritic cells is linked to phenotypic changes that may occur when these cells are kept in culture or subjected to various kinds of processing. This may lead to cell populations that are not very homogeneous and insufficiently characterized for therapeutic use.

Therefore, a real need exists for improving methods for sensitizing antigen presenting cells in order to increase the efficacy of these approaches and to broaden their applications, as well as to develop new means for vectorizing antigens or other molecules.

The present invention offers solutions to these questions. The goal of the present invention is to provide novels methods for sensitizing antigen presenting cells, especially dendritic cells, as well as for identifying, isolating, and characterizing novel membrane vesicles having notable immunogenic properties.

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More specifically, one of the aspects of the invention is to propose a novel, reproducible process for sensitizing antigen presenting cells by tumor antigens.

Another aspect of the invention is to propose a novel, reproducible process for sensitizing antigen presenting cells by tumor antigens, wherein it is not necessary that the tumor antigens be known.

Another aspect of the invention is to propose means for

establishing a tumor antigen bank.

Another aspect of the invention resides in lipid membrane vesicles, produced by the tumor cells or by the dendritic cells, that have immunogenic properties, as well as their use for producing antigen banks, sensitization of antigen presenting cells or antigen vectorization, specifically in the context of immunotherapy approaches.

For this purpose, an initial goal of the invention concerns a vesicle derived from tumor cells having the following characteristics:

- it is removed from its natural environment;
- it includes a lipid bilayer (referred to as "surface") that surrounds a cytosolic fraction,

and possibly,

- it has on its surface Class I and/or Class II major histocompatibility complex (MHC) molecules, possibly loaded with antigen peptides and/or adhesion molecules and/or lymphocyte costimulation molecules, and/or,
- it contains tumor antigen molecules and/or immunomodulators and/or chemoattractors and/or hormones and/or nucleic acids in its cytosolic fraction.

The secretion of vesicles by cells is a phenomenon described in the prior art (reticulocytes, B lymphocytes, macrophages). These vesicles are generally designated by the generic term "exosome," which reflects their mechanism of production via exocytosis of internal vesicles. However, the physiological role of these vesicles has not been truly established. Moreover, the structural

characteristics, properties, and functions of these vesicles vary according to the cell type from which they originate.

Unexpectedly, the inventors have now demonstrated that tumor cells are able to secrete vesicles that have especially interesting immunogenic properties. These vesicles generally correspond to an internal vesicle contained in a tumor cell endosome and secreted by the tumor cell after the external membrane of the endosome fuses with the cytoplasmic membrane of the tumor cell. Thanks to this formation mechanism, their cell of origin, and their original functional characteristics and properties, these vesicles are designated hereinafter by the term "texosome."

The expression "removed from it's natural environment" means that the vesicle is physically separated from the cell from which it originates, or that it is partially isolated or purified. Generally, the vesicle is therefore produced by the cell by exocytosis, then partially isolated or purified so as to obtain an enriched composition. This expression may also mean not only that the vesicle was secreted by the cell during fusion of the multivesicular endosomes with the plasmic membrane, but that it is no longer surrounded by the soluble elements that are in the lumen of the endosome, or that it is devoid of intact cells. The expression "derived from tumor cells" means that the vesicle has structural elements from a tumor cell. This vesicle is generally "derived" from a tumor cell in the sense that it is produced, at least partially, then released by a tumor cell, at a given stage

of its development.

According to an advantageous embodiment, the texosomes of the invention have MHC molecules loaded with antigen peptides and/or express adhesion molecules and/or express lymphocyte costimulation molecules, but have no antigenic tumor molecules, immunomodulators, or nucleic acids in their cytosolic fraction.

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According to another advantageous embodiment, the texosomes of the invention are such that the MHC molecules are "empty"; that is, not loaded with antigen peptides, and the texosomes include antigenic tumor molecules, immunomodulators and/or nucleic acids in their cytosolic fraction. Texosomes having empty MHC molecules may be obtained either from tumor cells that have, for example, a peptide transporter deficiency (PTD), or by washing texosomes or tumor cells in order to eluate the peptides associated with the MHC molecules.

According to an advantageous embodiment of the invention, the texosomes of the invention are such that the MHC molecules are loaded with antigen peptides and/or express adhesion molecules and/or are loaded with lymphocytic costimulation molecules and the texosomes contain antigenic tumor molecules, immunomodulators, and/or nucleic acids in their cytosolic fraction.

The term "tumor cells" generally encompasses any cell originating from a tumor, for example a solid or liquid tumor, as well as transformed or immortalized cells in vitro, preferably a solid, ascitic, or hematopoietic tumor.

By way of example, we may mention malignant melanoma-type cancer cells (coming from primary lines established "ex vivo," or dissociated cells from the operative specimen) that express peptides such as MART-1/Melan-A on their surface, in the MHC-Class I context, HLA-A 02-01, and containing the MART-1 protein antigen.

We may also mention cells originating from renal cancer (clear-cell adenocarcinoma) or from leukemias whose cells express specific translocation products.

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Therefore, the antigen peptides likely to load the MHC molecules originate, for example, from the following antigens: those from melanomas such as: MART-1, tyrosinase, MAGE-1/2/3, P53 (in various tumors), or Her2/Neu, PSA, CEA, or PSMA. Other tumor antigens are cited, for example, in the article by Rosenberg (Immunology Today 18 (1997), 175), incorporated by reference into the present document.

More generally, we may mention fusion/translocation products of oncogens or antioncogens, or differentiation antigens or self peptides or mutated peptides.

By "lymphocytic costimulation molecules," we designate, for example, molecules that give signals to T lymphocytes that are complementary to those given during interaction of Class I and II molecule peptide complexes with the T cell receptor.

We may mention, by way of example:

CD80, CD86, ICAM, LFA, CD40, certain members of the TNF R family and adhesion or chemoattractor molecules (enabling contact

between the professional antigen presenting cell and the effector lymphocytes, or the specific intracellular/localization transport ("trafficking/homing") of other cells to the vaccination or inflammation site).

The antigenic tumor molecules contained in the cytosol or presented by the texosomes come from proteins that are expressed selectively and/or abundantly by the tumor cells.

The immunomodulators that may be present in the cytosol of the texosomes are, for example:

- TNF-α, or
- Interleukin 1, or
- Interleukin 15, or
- C-CR (chemokines).

The nucleic acids likely to be present in the cytosol of the texosomes come from the tumor cell itself. These nucleic acids are located in the cytosol of the texosomes as a direct consequence of their formation mechanism. This may also involve heterologous nucleic acids.

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More specific characteristics of the texosomes of the invention are as follows:

- they are small membrane vesicles ranging from 60 to 100 nm approximately, most often ranging from 60 to 90 nm approximately, secreted by the tumor cells;
- they have molecules normally present in endosomes;
- they contain tumor antigens, such as, for example, MART-1 in the case of melanoma cells;

- they are devoid of dead cells and/or cellular debris;
- they are devoid of contaminants such as membrane contaminants, endoplasmic reticulum, Golgi apparatus, mitochondria or nucleus constituents;
- on their membrane, they have Class I/II functional molecules loaded with tumor antigen peptides;
- they may stimulate the proliferation of specific T lymphocytes in vitro;
- they may sensitize dendritic cells in vivo and in vitro that are able thereafter to activate the specific T cells of the tumor;
- when they are inoculated in vivo, especially intradermally, they are able to make established solid tumors regress;
- they have lymphocytic costimulation molecules such as CD40 and CD80, and/or;
- they contain the HSP70 ("heat shock") protein;
- they do not have the gp96 protein;
- they contain interleukins, chemoattractors, or immunomodulators.

Another interesting characteristic of the texosomes is that they contain phosphatidylserine in their outer layer. Phosphatidylserine (PS) is one of the main components of cellular membranes, and is normally present to a large extent in the inner layer of lipid bilayers. Under certain circumstances, such as the early stages of apoptosis, the PS is redistributed towards the outer layer. The presence of PS in the outer layer of the

cytoplasmic membrane of apoptotic cells constitutes a reconnaissance signal for macrophages. In order to determine whether the PS is exposed to the surface of the texosomes, preparations of purified exosomes starting from human melanoma FON cell supernatants were analyzed using the method described by Aupeix et al. (J. Clin. Invest. 99: 1546-1554, 1997). The phosphatidylserine content in the outer layer of the FON samples (containing 390 microg/ml of proteins) is 460 nM of PS. Therefore, the exosomes contain considerable quantities of PS in their outer layer.

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Tests for verifying that the texosomes of the invention have molecules that are normally present in endosomes consist of electronic microscopy and the immunoblot (Western Blot) test. These tests show that the texosomes of the invention express the transferin receptor, LAMP ("lysozome associated membrane protein") molecules, Class I/II molecules, and tumor antigens.

A test for verifying that the texosomes of the invention are free of contaminants is electronic microscopy and immunoblot with anticalnexin antibodies that is [sic] present in the endoplasmic reticulum.

A test for verifying that the texosomes carry functional Class I/II molecules loaded with tumor antigen peptides on their membrane consists of an antigenic presentation to specific T lymphocytes of the antigens of the tumor in question (proliferation tests of specific T clones of antigens and restricted Class I MHCs).

One may also use a test for cytokine (IFNγ, GM-CSF, TNFβ) secretion by the abovementioned T clones.

A test for verifying that there is in vivo and in vitro sensitization of dendritic cells capable of activating specific T cells of the tumor is given in Figure 7 (test of proliferation and/or secretion of cytokines by the specific antigen T clones, by the cross-priming method: texosomes from a MART-1+, HLA-A2- tumor loaded on a MART-1-, HLA-A2+ dendritic cell.

A test for verifying that the texosomes, when inoculated, specifically intradermally, are able to make established solid tumors regress in provided in Figure 6.

By way of example, an intradermal injection of 10 to 40 μg of tumor texosomes is made on the homolateral side of the established tumor from 3 to 10 days; the animal affected by the tumor is observed, as is the progressive disappearance of the established tumor over 7 to 10 days (in mouse-type rodents).

An advantageous texosome of the invention is constituted by a texosome as defined above and having on its surface Class I and/or Class II MHC molecules, possible loaded with antigen peptides and containing tumor antigen molecules in its cytosolic fraction. More specifically, the preferred texosome also has one or several lymphocytic costimulation molecules and/or the protein HSP70. In a specific embodiment, the texosome does not have the gp96 protein.

According to an advantageous embodiment, the invention concerns a texosome as defined above,

- expressing on its surface Class I and/or Class II major histocompatibility complex (MHC) molecules, and/or characteristic tumor antigens and/or lymphocytic/adhesion molecules and/or immunomodulators, and/or chemoattractors, which are exogenous in relation to the tumor cell from which the exosome is derived, or
- containing tumor antigens and/or immunomodulators and/or nucleic acids or cytotoxic agents or hormones that are exogenous in relation to the tumor cell from which the exosome is derived.

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The invention also involves a process for preparing texosomes as defined above. Advantageously, this process includes a stage for making available a biological sample and a stage for isolating texosomes from this sample.

The biological sample is advantageously composed of membrane fractions, cultured supernatants or supernatants from tumor cell lysates, or fresh tumor suspensions.

The biological sample may come from operative tumor specimens following surgical excision (1st case) or from tumor-bearing organs (surgically-excised organ) (2nd case), that is processed using mechanical dissociation (1st case) or by prolonged perfusion (2nd case).

The final cellular suspension is processed in the same way as the culture supernatants.

It may also involve cells processed by freezing/thawing in several successive cycles.

According to an advantageous embodiment, the biological sample used in the process of the invention is:

- a sample of efferent blood from the vein of the isolated organ affected by the tumor, or
- a sample of serum or plasma from the patient's circulating blood, or
- the product of drainage (physiological serum possibly containing dexamethasone, or cytotoxic agent simulating exocytosis of the texosomes) of a surgically-excised organ treated ex vivo by isolated perfused circuit for drainage of the tumor that it bears, or
- the supernatant of a tumor explant dissociated in vitro.

The sample of efferent blood from the isolated tumor-affected organ corresponds to 20 to 50 ml of blood from the main efferent vein of the tumor-affected organ, sampled prior to surgical ablation intervention.

The drainage product from a surgically-excised organ and treated ex vivo by isolated perfused circuit occurs [sic] as follows.

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In the case of an organ having an afferent artery and an efferent vein, the artery is characterized [sic: presumably "catheterized"] by a plastic tube connected to a pouch containing physiological serum and other agents, if applicable. The organ is drained and the liquid leaves by another, descending tube catheterizing the vein (for example, in the case of kidney cancer or a cerebral glioblastoma).

Dexamethazone, which may be contained in the drainage product, is used to increase cellular stress and exocystosis of texosomes out of the tumor cell.

The supernatant of a tumor explant dissociated in vitro is obtained as follows:

- we proceed with mechanical dissociation of the tumor, leading to a unicellular suspension containing tumor cells, cells from the tumor stroma, and immune system cells; this suspension may irradiated and recovered for differential ultracentrifugations.

As indicated above, in a specific implementation mode of the process of the invention, the biological sample may be treated by one or several agents for stimulating texosome production. This treatment may include the addition of steroid agents (dexamethazone, for example), pharmacological agents (for example, cytotoxic agents such as taxanes, cisplatinum, etc.), agents likely to increase the quantity of multivesicular endosomes, and/or irradiation of the sample.

As regards irradiation, it must be sufficient to cause cytostatic action in the tumor cells. Irradiation of the tumor cells may be performed before the cells are cultured, or during or after the tumor cells are cultured. Additionally, it is advisable to irradiate when the tumor cells are alive, that is:

- either on the excised tumor-bearing organ prior to perfusion,
- or on the cultured cells,

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- or on the mechanically-disassociated cellular suspension; but, in any case, before tumor cell dysfunction due to hypoxia/vascular necrosis/dehydration.

As concerns steroid treatment, it creates cellular activation leading to exocytosis of the texosomes.

As concerns treatment using pharmacological agents, this approach:

- modifies the cytoskeleton and rearranges intracellular compartments, disturbing the phenomena of internalization and exocytosis,
- depolymerizes microtubules.

As concerns treatment using an agent that increases the quantity of multivesicular endosomes, this occurs during culturing of the cells; as an agent, we may mention nocodazole (a drug that depolymerizes microtubules), bafilomycin (a drug that inhibits vacuolar ATPases) ("Bafilomycins: A class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells" (1988) Proc. Natl. Acad. Sci. USA 85: 7972-7976).

An advantageous process for preparing texosomes according to the invention is performed:

a) either starting from tumor cell cultures, and including:

- an irradiation, at sufficient intensity to cause cytostatic action in the tumor cells and not exceeding 15,000 rads, advantageously at around 10,000, of the tumor cells before, during, or after their culturing, or
- a treatment, during culturing, of the tumor cells using steroids, dexamethazone for example, or cytotoxic agents,

such as 5-fluorouracil (5 Fu) or cis-platinum, docetaxel, anthracycline, spindle poison, antipyrimidic, or interleukin, for example IL10, IL2, IL15, GM-CSF, or

- treatment with agent that increases the quantity of multivesicular endosomes, such as nocodazole (Gruenberg J. et al., (1989) "Characterization of the Early Endosome and Putative Endocytic Carrier Vesicles in vivo and with an Assay of Vesicle Fusion in vitro" The Journal of Cell Biology 108: 1301-1316), thereby increasing texosome production,

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b) or from a sample of physiological serum draining from a surgically-excised organ and treated ex vivo by isolated perfused circuit for drainage of the tumor that it bears, or

c) from a supernatant of a tumor explant dissociated in vitro, including:

- steroid treatment, using dexamethazone, for example, or treatment with cytotoxic agents, such as 5-fluorouracil (5-Fu); cis-platinum, taxanes, or interleukin; IL-10, IL-2, GM-CSF, for example.

The texosome isolation stage may be carried out using various techniques such as centrifugation, chromatography, elelctrophoresis, nanofiltration, etc. It may involve, for example, differential centrifugation of membrane fractions of culture supernatants or tumor cell lysates or fresh tumor suspensions, and recovery of the fraction or fractions containing

the exosomes (Raposo et al., J. Exp. Med. 1996, 183: 1161-1172). In this implementation mode, the membrane fraction of supernatants is the one obtained following ultracentrifugation at 100,000 g. Advantageously, it may involve electrophoresis in the fluid phase, which enables the separation of biological materials according to their load. The following examples show that this technique may be advantageously used to isolate texosomes with good yields. This technique is also especially advantageous on the industrial level.

Another goal of the invention is a texosome preparation process as defined above, also including:

- either genetic modification of the tumor cells by exogenous genes coding for Class I and/or Class II MHC molecules, and/or genes coding for antigens characteristic of tumors and/or genes coding for costimulation/adhesion molecules or attractor chemokines, with the products of these exogenous genes possibly being expressed on the surface of the texosomes and/or being sequestered inside the texosomes,

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- or in vitro modification of the texosomes produced by the tumor cells, such as introducing (via electroporation, fusion with a synthetic liposome, recombinant virus, or chemical method) proteins or nucleic acids or pharmaceutically-defined drugs into and/or with the texosomes.

The invention also concerns texosomes obtained using the process described above.

The texosomes of the tumor cells transfected as described above are collected and used as tumor vaccines.

The texosomes modified in vitro as indicated above are intended to deliver the exogenous material to a target cell in vitro or in vivo.

As regards fusion with a synthetic liposome, this process is carried out as indicated, for example, in Nabel et al. (1996) or in Walker et al. (1997, Nature 387, pages 61 ff.).

The invention also concerns antigen presenting cells, in particular B lymphocytes, macrophages, monocytes, or dendritic cells, loaded with texosomes as defined above. Dendritic cells are advantageously used.

The dendritic cells of the invention have the following characteristics:

- in models of tumors that do not express Class I molecules and therefore are not able to stimulate CD8+ T cells, dendritic cells loaded with texosomes from tumor cells may present these tumor peptides to cytotoxic T cells in the context of Class I MHC molecules (characteristic no. 1);
- dendritic cells loaded with tumor cell texosomes that are injected intravenously or subcutaneously are also very effective (characteristic no. 2).

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A test for demonstrating characteristic no. 1 is as follows.

In the human system, a negative Class I texosome, incubated in the presence of a positive Class I dendritic cell, may enable stimulation of specific CD8+T clones of the antigen contained in

the texosome (see Figure 7).

A test for demonstrating characteristic no. 2 is as follows.

In a murine system where the tumor is ranked as I negative and where the texosomes are also devoid of Class I molecules, these texosomes may, when incubated and loaded onto the dendritic cells, mediate an antitumor immune response, whereas they cannot do so on their own following intradermal injection.

The invention also concerns a process for preparing antigen presenting cells as defined above, including the incubation stage of antigen presenting cells in the presence of texosomes as defined above, and the stage for recovering the antigen presenting cells loaded with the texosome.

The invention also concerns presenting cells loaded with texosomes and likely to be obtained by the process described above.

Another goal of the invention is use of texosomes as defined above for sensitizing antigen presenting cells, especially B lymphocytes, macrophages, monocytes, or dendritic cells, or for stimulating specific T lymphocytes.

The invention also concerns a membrane vesicle removed from its natural environment, secreted by texosome-loaded antigen presenting cells as defined above.

In order to obtain the membrane vesicles defined above, one may use a process including:

- a stage for preparing a texosome as defined above;

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- a stage for incubating a texosome with antigen presenting

cells;

- a stage for differential centrifiguation of membrane fractions of cultured supernatants or lysates of the texosome-loaded antigen presenting cells and
- a stage for recovering the fraction containing the membrane vesicles.

Another goal of the invention is membrane vesicles as described above and likely to be obtained via the process described above.

On this topic, the invention concerns membrane vesicles produced by dendritic cells. Unexpectedly, the inventors have demonstrated that dendritic cells are able to produce membrane vesicles having especially advantageous immunogenic properties. Such vesicles have been visualized, isolated, and characterized starting from culture supernatants of dendritic cells, specifically immature human dendritic cells. Unlike the vesicles described thus far, these vesicles are completely advantageous in that they present, in a major and simultaneous fashion, Class I and II major histocompatibility complex molecules. These membrane vesicles include a lipid bilayer surrounding a cytosolic fraction, and are designated hereinafter by the term "dexosome" due to their origin and their original biochemical and biological properties. These vesicles have remarkable immunogenic properties, since they are able to stimulate the production and activity of cytotoxic T lymphocytes, both in vitro and in vivo, and since they suppress in vivo the growth of established tumors, in a manner that is dependent on the T lymphocytes and limited to

the MHC type. Dexosomes therefore constitute active ingredients that are especially well-suited to non-cellular immunotherapy approaches.

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Specific membrane vesicles according to the invention are therefore vesicles that can be produced by dendritic cells, and that have one or several Class I major histocompatibility complex molecules and one or several Class II major histocompatibility complex molecules.

The dexosomes advantageously include lymphocyte costimulation molecules, specifically the molecules CD63 and/or CD82 and/or CD86, preferably at least CD86. The studies presented in the examples show that the dexosomes are strongly marked by antibodies directed specifically against these costimulation molecules.

Additionally, electronic microscopy analyses show that the dexosomes are homogeneous and have a diameter ranging from 60 to 100 nm approximately, generally from 60 to 90 nm approximately.

An especially preferred variation of the invention is therefore represented by a dexosome whose diameter ranges from approximately 60 to 90 nm, obtained from a dendritic cell, and having:

- one or several Class I major histocompatibility complex molecules;
- one or several Class II major histocompatibility complex molecules;
- one or several CD63 molecules;

- one or several CD86 molecules; and
- one or several CD82 molecules.

In a specific embodiment of the invention, the dexosomes also include one or several antigen peptides and/or are obtained from immature dendritic cells.

Still according to a specific embodiment, the dexosomes are devoid of H2-M, Li chain, and calnexin markers (the latter being a specific endoplasmic reticulum marker).

Additionally, still according to an advantageous embodiment, the dexosomes of the invention also include phosphatidylserine (PS) in their outer layer. Thus, preparations of purified exosomes from supernatants of dendritic cells derived from bone marrow were analyzed using the method described by Aupeix et al. (J. Clin. Invest. 99: 1546-1554, 1997). The phosphatidylserine content in the outer layer of the BMDC samples (containing 35 microg/ml of proteins) is 80 nM of PS. The dexosomes therefore contain large quantities of PS in their outer layer.

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The dexosomes may be prepared according to a methodology including an initial stage for obtaining dendritic cells or a cell culture containing dendritic cells, a second optional stage, during which the cells may be sensitized to antigens of interest, and a third stage including the production of dexosomes from cell cultures. These various stages may be advantageously carried out according to the methodologies described hereinafter.

Preparing Dendritic Cells

The first stage of the process includes development of a

dendritic cell culture or cultures. This may involve cell cultures enriched with dendritic cells, or cell cultures having mainly dendritic cells. Advantageously, these are obviously human dendritic cells.

Dendritic cell preparation is well-documented in the literature. Hence, it is known that these cells may be obtained from immune system stem cells or from monocyte precursors, or they may be isolated directly in differentiated form (Review by Hart, Blood 90 (1997) 3245).

Obtaining dendritic cells from stem cells is illustrated, for example, by Inaba et al. (J. Exp. Med. 176 (1992) 1693), Caux et al. (Nature 360 (1992) 258) or Bernhard et al. (Cancer Res. 55 (1995) 1099). This research shows that dendritic cells may be produced by culturing bone marrow in the presence of Granocyte Macrophage Colony Stimulating Factor (GM-CSF) or, more specifically, from hematopoietic stem cells (CD34+) by culturing in the presence of a combination of cytokines (GM-CSF + TNFα).

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Obtaining dendritic cells from monocyte precursors is illustrated, for example, by Romani et al. (J. Exp. Med. 180 (1994) 83), Sallusto et al. (J. Exp. Med. 179 (1994) 1109), Inaba et al. (J. Exp. Med. 175 (1992) 1157) or Jansen et al. (J. Exp. Med. 170 (1989) 577). These methodologies are essentially based on sampling mononucleated cells in blood and culturing them in the presence of various combinations of cytokines. A specific method consists of processing monocyte precursors from blood in the presence of combinations of cytokines, such as Interleukin-4

+ GM-CSF or Interleukin-13 + GM-CSF, for example. This technique is also illustrated by Mayordomo et al., 1995. It is also possible to process monocyte precursors with pharmacological cell differentiation agents, such as calcium channel activators.

Another approach for obtaining dendritic cells consists of isolating already-differentiated dendritic cells from biological samples. This approach was described, for example, by Hsu et al. (Nature Medicine 2 (1996) 52). The methodology described by this team mainly consists of harvesting peripheral blood samples and processing them with various gradients and centrifugations in order to extract the dendritic cells from them.

The preferred methodology in the context of the present invention is based on producing dendritic cells from monocyte precursors or from bone marrow. These methodologies are illustrated in the examples. More specifically, we prefer, in the context of the present example, to use dendritic cells obtained by processing monocyte precursors (contained in blood or in marrow) in the presence of a GM-CSF+IL-4 or GM-CSF+IL-13 combination.

Additionally, for implementing the present invention, it is especially advantageous to use a population of dendritic cells that includes immature dendritic cells. Advantageously, one uses a population of dendritic cells that is mainly composed (i.e., at least 60%, preferably 70%) of immature dendritic cells. The immature condition of the dendritic cells corresponds to an early stage of their development, during which they have high endocytic activity and express low levels of Class I and II MHC molecules

and lymphocyte costimulation molecules on their surface. Surprisingly, the inventors have found that only immature dendritic cells were able to produce a significant quantity of membrane vesicles. This discovery is all the more surprising given that immature dendritic cells are known for their weak ability to stimulate T lymphocytes, and therefore for their low level of biological activity (Cella, Nature London, 388 (1997) 782).

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The first stage of the process of the invention may therefore advantageously include the preparation of a dendritic cell population including immature dendritic cells, especially from monocyte precursors, and more specifically by processing them with a combination of cytokines such as GM-CSF+IL-4 or GM-CSF+IL-13.

It is also possible to use, in the context of the present invention, populations of immortalized dendritic cells. These may be lines of immortalized dendritic cells (line D1 used in the examples, or any other line produced, for example, by introducing the myc oncogene into the dendritic cells). They may also be dendritic cells that are prepared, then immortalized in vitro. The advantage of using immortalized dendritic cells resides in the constitution of banks of cells that are sensitized to various antigen groups, and that are usable in industry for preparing dexosomes likely to be administered to entire families of patients.

When the dendritic cells are prepared, they may be kept in

culture, further purified, stored, or used directly in the following stages of the process.

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Sensitizing the Dendritic Cells

The dexosomes of the invention may be prepared from non-antigen-loaded dendritic cells, that is, cells not having determined antigens in their membranes or cytosol. Such dexosomes are then designated as "primitive" or "virgin."

According to a preferred implementation mode, the dexosomes of the invention are, however, prepared from dendritic cells that have been sensitized to an antigen or to a group of antigens. In this embodiment, the dexosomes themselves bear the antigen(s) and are therefore able to induce a response to them.

Various techniques may be used to sensitize the dendritic cells to antigens. These techniques were mentioned above and include:

- bringing the dendritic cells into contact with antigen peptides ("peptide pulsing"). This approach consists of incubating the dendritic cells for a variable period (generally from 30 minutes to 5 hours, approximately) with one or several antigen peptides; that is, with a peptide originating from an antigen, as might result from processing the antigen with a presenting cell of the antigen. This type of approach was described, for example, for antigen peptides of the HIV virus, Influenza, or HPV, or for peptides derived from the Mut1, Mart, Her2 or Neu antigens, for example (Macatonia et al., J. Exp. Med. 169 (1989) 1255; Takahashi

et al., Int. Immunol. 5 (1993) 849; Porgador and Gilboa, J. Exp. Med. 182 (1995) 255; Ossevoort et al., J. Immunother. 18 (1995) 86; Mayordomo et al., cited above; Mehta-Damani et al., J. Immunol. (1994) 996). It is also possible to incubate the dendritic cells with an acid peptide eluate from a tumor cell according to the methodology described by Zitvogel et al. (1996; cited above).

- bringing the dendritic cells into contact with one or several antigens ("antigen pulsing"). This approach consists of incubating the dendritic cells not with one or several antigen peptides, but with the intact antigen or antigens. The advantage of this technique resides in the fact that the antigen will be transformed into antigen peptides by the natural mechanisms of the dendritic cell, such that the resulting antigen peptides presented by the dendritic cell should have greater immunogenicity. This approach was illustrated, for example, by Inaba et al. (J. Exp. Med. 172 (1990) 631) or by Hsu et al., (Nature Medicine 2 (1996) 52). /23

- bringing the dendritic cells into contact with one or several antigen protein complexes. This approach is similar to the preceding approach, but may improve the antigen's transformation and/or presentation efficacy. Specifically, the antigen may be used in soluble form or complexed with target elements, enabling targeting of membrane receptors such as mannose receptors or immunoglobulin receptors (Rfc). It is also possible to make the antigen in particulate form

in order to improve its penetration or its phagocytosis by the cells.

- Bringing the dendritic cells into contact with cells or cell membranes expressing antigens or antigen peptides. This technique is based on the direct transfer of antigens or antigen peptides by fusion of cells or cell membranes. This approach was illustrated, for example, by the fusion of dendritic cells with tumor cell membranes (Zou et al., Cancer Immunol. Immunother. 15 (1992) 1).
- Bringing the dendritic cells into contact with membrane vesicles containing antigens or antigen peptides (specifically, exosomes of tumor cells as described above). This approach to sensitizing dendritic cells using exosomes, as demonstrated in the present invention, is especially advantageous because it does not require knowledge of specific antigens and the loaded antigen peptides are in their native conformation. This technology is illustrated in the examples.

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- Bringing dendritic cells into contact with liposomes containing antigens or antigen peptides (Nair et al., J. Exp. Med. 175 (1992) 609).
- Bringing dendritic cells into contact with RNAs coding for antigens or antigen peptides (see Boczkowsky et al., 1996, cited above).
- Bringing dendritic cells into contact with DNAs coding for antigens or antigen peptides (possibly incorporated into

plasmid-, viral-, or chemical-type vectors). Thus, a method for sensitizing dendritic cells consists, for example, of infecting dendritic cells with a virus against which protection is being sought. This was described, for example, for the Influenza virus (Bhardwaj et al., J. Clin. Invest. 94 (1994) 797; Macatonia et al., cited above). Another approach consists of delivering, by means of a virus or other nucleic acid transfer vectors, a DNA coding for the antigen(s) or antigen peptides of interest. Such an approach was illustrated, for example, by Arthur et al. (Cancer Gene Therapy, 1995) or by Alijagie et al. (Eur. J. Immunol. 25 (1995) 3100). Certain viruses, such as adenoviruses, AAVs, or retroviruses, are apparently able to be used for this purpose, in order to deliver a nucleic acid into a dendritic cell.

Preferred techniques in the context of the invention are sensitization methods that use membrane vesicles (of the exosome type), antigen peptides, vectors, RNAs, or tumor peptide acid eluates (PAE). The use of membrane vesicles as well as "peptide pulsing" and the PAE method are illustrated in the examples and are especially preferred.

Producing Dexosomes

Once the populations of dendritic cells are obtained and, if applicable, sensitized to one or several antigens, dexosomes may be prepared.

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This preparation includes an initial optional stage, for

treating the cells, followed by a second stage for isolating the dexosomes.

The first stage for treating the cells results from the inventors having demonstrated that the production of dexosomes by dendritic cells is a regulated phenomenon. Thus, in the absence of treatment, the quantities of dexosomes produced are relatively low. Specifically, when one uses a population of mature dendritic cells that have not been previously stimulated, the production of dexosomes is practically undetectable. Therefore, the inventors have shown that dexosome production essentially depends upon the type of dendritic cells and the treatment of these cells. These are the required elements for obtaining dexosomes with advantageous properties in quantities permitting industrial use. Treatment of dendritic cells is therefore advantageously performed in such a way as to stimulate the production of dexosomes by these cells. This stimulating treatment may be carried out either by culturing the cells in the presence of certain cytokines or by irradiating the cells, or by lowering the culture's pH, or by combining these various types of treatment.

In the first implementation method, the dendritic cells are incubated in the presence of a cytokine preferably selected from among gamma interferon (IFNγ), interleukin-10 (IL-10), and interleukin-12 (IL-12), preferably gamma interferon and IL-10. As the examples illustrate, these cytokines appear to have a quite pronounced stimulating effect on dexosome production (factor 3 to 5). Moreover, surprisingly, no stimulating effect was observed in the presence of the following cytokines: IL-1β, IL-2, IL-4, IL-6,

and IL-15, and an inhibiting effect was even observed in the presence of lipopolysaccharide (LPS) or TNFα, which are described as being stimulants of dendritic cell maturation. Therefore, these results show (i) the regulated character of dexosome production, and (ii) the specific effect of certain cytokines on this production. Moreover, these results illustrate the surprising benefit of using these immature dendritic cells, as well as of using, in the stimulation stage, cytokines that induce an immature state of the cells, such as IL-10. In this implementation method, the cytokines are used in doses that are adaptable by the expert depending upon (i) the cytokine, (ii) the cell population, and (iii) the possible realization of other forms of processing. It goes without saying that the cytokines are preferentially used in subtoxic doses. The doses of interleukin generally range from 1 to 100 ng/ml, preferably from 1 to 50 ng/m. Interferon may be used in doses ranging from 1 to 500 IU/ml, preferably from 5 to 200 IU/ml.

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In the second implementation method, the dendritic cells undergo irradiation. The results presented in the examples show that irradiating the cells also increases the level of dexosome production. Irradiation is generally performed at between 1000 and 5000 rads, preferably between 2000 and 4000 rads, advantageously at around 3000 rads.

The second stage involves isolating the dexosomes. This stage has the objective of separating the dexosomes from the dendritic cells and/or from the culture medium. This stage makes

it possible to obtain a dexosome-enriched composition that is essentially devoid of intact cells. Preferentially, this stage yields a composition including at least 70% dexosomes, preferably at least 85%.

Isolating the dexosomes can be carried out by using various techniques for separating biological materials. As was described previously for tumor cell texosomes, these techniques may be based on differences in size, mass, load, or density of the dexosomes.

Thus, the dexosomes can be isolated by centrifugation of the culture medium or of the culture supernatant or of membrane fractions or of dendritic cell lysates. This may involve, for example, differential centrifugation and/or density gradient centrifugation, followed by recovery of the fraction(s) containing the dexosomes. This type of methodology is based on separating, via successive centrifugations, membrane vesicles, on the one hand, and cells, cell debris, internal vesicles, etc. on the other. In this particular implementation method, the fraction containing the dexosomes is generally the one obtained after ultracentrifugation at 100,000 g. This method is illustrated in Examples 1 and 8.

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The dexosome isolation stage may also be carried out using chromatography, electrophoresis, and/or nanofiltration.

Advantageously, it may involve electrophoresis in the fluid phase and/or density gradient electrophoresis. Electrophoresis in the fluid phase, which enables separation of biological materials

according to their load, is highly advantageous. Example 11 below shows that this technique may be advantageously used for isolating exosomes with good yields. This technique is also especially advantageous on the industrial level.

Purification by chromatography may also be involved. In particular, we may mention ion exchange chromatography, gel permeation (or exclusion) chromatography, or hydrophobic chromatography. Given the lipidic nature of the dexosomes, ion exchange chromatography is especially interesting. Nanofiltration may be performed according to known techniques starting from a cell supernatant.

Using chromatography and/or electrophoresis and/or nanofiltration techniques constitutes another important aspect of the present invention, since this enables, as compared to currently-used technology, higher-quality production in quantities adapted for industrial use (for pharmacological uses, in particular).

On this topic, the invention also concerns a process for preparing membrane vesicles that includes at least one separation stage using electrophoresis, chromatography, or nanofiltration. This process is more specifically adapted to preparing exosome-type membrane vesicles, such as texosomes or dexosomes. In this process, the separation stage using electrophoresis or chromatography may be performed directly on a culture supernatant, a cell lysate, or a prepurified preparation. The type of electrophoresis used is preferably electrophoresis in the fluid phase.

Dexosomes have remarkable properties that are illustrated in the examples. Dexosomes stimulate proliferation of cytotoxic T lymphocytes in vitro. Moreover, in vivo, dexosomes are able to block tumor growth. These vesicles are therefore able to present, in a very efficacious manner, in association with Class I and II MHC molecules, antigens of great interest. Dexosomes therefore have many applications; in the areas of cancer and infectious or parasitic diseases, for example. Additionally, at high doses (likely to induce tolerance), dexosomes may also be used in treating pathologies such as allergies, asthma, or autoimmune diseases. Moreover, "primitive" dexosomes may also be used as an adjuvant to stimulate and/or modulate an immune response.

Another goal of the invention is the use:

- of texosomes as defined above, or
- of antigen presenting cells as defined above, or
- of dexosomes as defined above,

for the stimulation and possible in vitro amplification of specific T lymphocytes of antigens contained in the texosomes, antigen presenting cells, or dexosomes, - or of B lymphocytes, specifically for the stimulation and in vitro amplification of T lymphocytes.

The invention also concerns the use of texosomes as defined above, or of antigen presenting cells as defined above, or of dexosomes as defined above, for the ex vivo selection of a repertoire of T lymphocytes that are likely to recognize specific antigens contained in the texosomes, antigen presenting cells, or

dexosomes.

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Another goal of the invention is a drug including as an active ingredient at least one texosome as defined above, one antigen presenting cell as defined above, and/or one dexosome as defined above, in association with a pharmaceutically-acceptable vehicle.

Advantageously, the invention concerns a drug as defined above for use in treating cancer and infectious or parasitic diseases.

More preferably, the drug includes texosomes or dexosomes as defined above.

According to another implementation method, the invention concerns a drug as defined above for use in treating allergy-type, asthma, or autoimmune disease pathologies.

As an appropriate galenic form, the texosomes or dexosomes may be contained in physiological serum, in a vial or any other appropriate means (syringe, pouch, etc.). They may be prepared extemporaneously or stored, i.e. in frozen form at -80 degrees C. The solutions used may composed of saline solutions, possible supplemented with stabilizing agents and/or adjuvants. The stabilizing agents may be proteins or high-molecular-weight molecules. More specifically, we may mention proteins such as human albumin serum, or molecules such as dextran or poloxamer, for example.

The compositions of the invention may also include or be used in association with one or several adjuvants. The adjuvant

may be any immunostimulating pharmacological agent, such as a cytokine, for example (specifically, interleukin-12). Such agents are traditionally used in clinical protocols or in vaccine compositions. Additionally, the adjuvant according to the invention may also be an agent capable of stimulating the production of dendritic cells in vivo. By way of example, we may mention the compound Flt3. The combined use of this type of agent increases the number of dendritic cells and therefore potentially improves the efficacy of the compositions of the invention.

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Another goal of the invention therefore concerns an association of texosomes and/or dexosomes with an adjuvant, with a view towards use that is simultaneous, separate, or occurs at various time intervals.

An appropriate mode of administration of the drugs of the invention is constituted by injections, specifically intradermal or subcutaneous injections. This mode of administration is especially suitable when the active ingredient of the drug is constituted by texosome- or dexosome-loaded dendritic cells.

Appropriate dosages range from 0.01 to 10, and particularly from 0.10 to 5, even more preferably from 0.15 to 2 µg/kg of body weight, and 10 µg for intradermal reaction tests.

The drugs of the invention may also be used at 100 µg for prophylactic vaccination treatments.

The goals targeted by using the drugs of the invention are:

- delayed hypersensitivity (tests in cancer patients), or

- prophylactic therapy, or
- use in the context of detecting the frequency of specific cytotoxic lymphocyte or interferon secretor precursors by the limit dilution technique.

This involves using autologous or allogenic dendritic cells that have been preincubated with the texosomes of the invention, as targets of peripheral lymphocytes of tumor-bearing subjects, before, during and after anti-tumor treatment (traditional treatment or specific active immunization).

The invention also concerns the use of a texosome as defined above, or a an antigen presenting cell as defined above, or of dexosomes as defined above, for the preparation of a drug intended to treat tumors, specifically solid, ascitic, and hematopoietic tumors.

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As examples of solid tumors, we may mention: kidney cancer, breast cancer, colon cancer, lung cancer, stomach cancer, liver cancer, melanomas, sarcomas, etc.

As examples of hematopoietic tumors, we may mention: leukemias and malignant Hodgkin's or non-Hodgkin's lymphomas.

As indicated above, the compositions of the invention, specifically compositions including dexosomes, may also be used for treating infectious or parasitic diseases. For this type of application, the dexosomes are loaded with antigens or peptides of the infectious agent (virus) or of the parasite.

The invention also concerns the use of a texosome as defined above, or of a dexosome as defined above, for testing delayed

hypersensitivity of the cancer or as a diagnostic research tool on the frequency of specific cytotoxic CTL precursors.

The invention also concerns the use of a texosome, or of a fraction or of a constitutive component of a texosome as defined above, or of a dexosome as defined above, for the transfer of biological material into a cell in vitro or in vivo.

The invention also concerns the creation of banks of texosomes that are derived from tumor cells having a common or different histological type.

The latter are composed of mixtures of texosomes made from tumor cell lines for a given type of cancer. These texosome banks may make it possible to sensitize antigen presenting cells, specifically dendritic cells, against all tumors of this type.

The invention also concerns mixtures of texosomes or dexosomes.

We may mention, for example, mixtures of texosomes for genetically-linked tumors (ovarian and breast cancer) or presenting known p53, p16 mutations (breast cancer, sarcoma).

We may also mention mixtures of tumor texosomes with vesicles originating from cells that are immortalized and transfected in order to express costimulation molecules, adhesion molecules, attractor chemokines (different from those expressed on the texosomes).

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The present invention will be described in greater detail by means of the following examples, which must be considered illustrative and non-limiting.

DESCRIPTIONS OF THE FIGURES

Table 1. The tumor line cells were incubated for 24 h at a density of one million cells per milliliter. The texosomes were then prepared (see example) from culture medium via differential ultracentrifugation. The texosomal protein concentration is measured using the Bradford test (BioRad Protein Assay [BioRad]).

MZ-2 is described in Traversari et al., (1992).

The asterisks mean that the various primary lines were established and characterized in the clinical biology laboratory at the Institut Gustave Roussy and are accessible upon request.

Figures 1A and 1B. Morphology of the multivesicular endosomes and of the texosomes derived from TS/A cells.

A. Ultrafine sections of TS/A cells analyzed by electronic microscopy. Detail of the cytoplasm showing an endosomal compartment containing vesicles that are 60-80 nm in diameter.

B. Preparation of texosomes from TS/A cells analyzed using electronic microscopy using the intact vesicle technique (Raposo et al. (1996)). The texosome preparations contain a majority population of vesicles that are 60-80 nm in diameter, and whose size and morphology are similar to the internal vesicles of the multivesicular endosomes shown in A.

Figure 2. Presence of various markers in the tumor cell texosomes.

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A. Two micrograms of texosomal proteins (Exos) or 2 X 10^5 tumor cells were analyzed using the Western Blot test by means of specific monoclonal antibodies of: Class I MHC molecules

(Machold, Robert P. et al. (1995) "Peptide Influences the Folding and Intracellular Transport of Free Major Histocompatibility Complex Class I Heavy Chains" J. Exp. Med. 181: 1111-1122), of transferin receptors (TfR) (with the corresponding antibody being H68.4, described in Biochimica et Biophysica Acta (1992) 1136(1): 28-34), Lamp 1 and 2 (rat anti-mouse monoclonal antibodies, Pharmingen) and calnexin (Hebert, Daniel N. et al. (1995) "Glucose Trimming and Reglucosylation Determine Glycoprotein Association with Calnexin in the Endoplasmic Reticulum" Cell 81: 425-433).

B. Ten micrograms of texosomal proteins from a melanoma (FON) cell line or 10 μg total proteins of the same cells were analyzed by Western Blot using an anti-MART-1 antibody (Marincola, F. et al. (1996) "Analysis of Expression of the Melanoma Association Antigens MART-1 and gp100 in Metastatic Melanoma Cell Lines and "In Situ" Lesions" Journal of Immunotherapy 19: 192-205).

C. The texosomal proteins of a line of melanoma (FON) cells or the total proteins of the same cells were analyzed by Western Blot using an anti-HSP antibody.

D. The texosomal proteins of a line of melanoma (FON) cells or of the MZ-2 line were analyzed by Western Blot using an anti-gp96 antibody.

Figure 3. The texosomes derived from a positive (FON) MART-1 tumor line stimulate a specific MART-1 T clone.

Twenty thousand cells from the LT8 (or LT12, results not shown) T clone were incubated with texosomes derived from FON

(MART-1 and positive HLA-A2 cell lines) or from GIAM (cells from a Mart-1 negative nephroma line) as a negative control for 48 h. TNFβ production by the T clone cells was measured by biological assay with WEHI cells (Espavik et al.). The texosomes induce the production of IFNγ by the T clone, thus revealing the presence of HLA-A2/peptide complexes derived from MART-1 on the surface of the texosomes.

- LT8+TexGIAM" corresponds to LT8 T clones incubated in the presence of texosomes derived from GIAM tumor cells;
- LT8+TexFON" corresponds to LT8 T clones incubated in the presence of texosomes derived from FON tumor cells;
- "LT8+TumFON" corresponds to LT8 T clones incubated in the presence of texosomes derived from FON tumor cells;

In the x-coordinates, we showed the conditioned cells and ordered the quantity of TNFβ produced (pg/ml).

Figure 4. The texosomes from P815 cells expressing βGal stimulate splenocytes from mice immunized with recombinant βGal adenoviruses.

Splenocytes (10^5) from BALB/c mice immunized 2 months prior with 10^6 pfu recombinant βGal adenovirus having rejected a tumor expressing βGal were incubated with texosomes derived from P815 cells (white diamonds ◊) or from P815-βGal cells (black squares |). The splenocytes not incubated in texosomes provide the "background noise" symbolized by black dots (?). After 5 days of culturing, 1μCi of tritiated thymidine was added per culture well. The incorporation of tritium into the cellular DNA was

measured 18 h later. Results that were significantly different according to Fisher's exact method are marked *.

In the x-coordinates, we showed the quantity of texosomes derived from P815 tumor cells (μg/ml) and ranked the counts per minute (CPM).

Figure 5. The MART-1 tumor antigen contained in texosomes may be presented to the T lymphocytes by dendritic cells.

Increasing does of texosomes derived from FON tumor cell lines (MART-1+, HLA-A2+, A1-) and from MZ2 tumor cell lines (MART-1+, HLA-A2-, A1+) were incubated (20,000 cells per 96-microplate well) with the LT12 T clones (HLA-A2/MART-1 specific) in the presence of HLA-A2+ dendritic cells derived from circulating macrophages (DCA2) (Sallusto, F. and Lanzavecchia, A. 1994, Efficient Presentation of Soluble Antigen by Cultured Human Dendritic Cells is Maintained by GMCSF and IL-4 and Downregulated by TNFα. J. Exp. Med. 179: 1109-1118). The secretion of IFNγ represented in y-coordinates (pg/ml) was measured in the culture supernatants after 2 days. The FON-derived texosomes, as well as the MZ2-derived texosomes, induced secretion of IFNγ by LT12 and LT8 (results not shown). The FON cells also induced a strong secretion of IFNγ by the T clones, whereas the MZ2 cells, which do not express the adequate HLA molecule haplotype (HLA-A2), did not induce IFNγ production.

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- "LT12+DCA2" corresponds to LT12 T clones incubated in the presence of HLA-A2+ dendritic cells;
- "LT12+DCA2+TexFON" corresponds to LT12 T clones incubated in

the presence of dendritic cells loaded with texosomes derived from FON tumor cells;

- "LT12+DCA2+TexMz2" corresponds to TL12 [sic] clones incubated in the presence of dendritic cells loaded with texosomes derived from MZ2 tumor cells.

Figures 6A and 6B. Anti-tumor effects of the texosomes in vivo.

One hundred thousand TS/A tumor cells were injected per Balb/c(A) or Nude (B) mouse. Three days later, each mouse received two successive intradermal injections 24 h apart (represented by J3 and J4 in the figure) of 20 µg to 30 µg of texosomes. The size of the tumors was then measured twice weekly. Statistical analyses were performed using Fisher's exact method (95% significance is indicated by a *).

A. Two groups of 5 mice received texosomes derived from TS/A (black triangles) or from MCA38 (white triangles Δ) (a colon adenocarcinoma line derived from a C57BL/6 mouse), as a negative control. Only the TS/A-derived texosomes have an anti-tumor effect in vivo.

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B. Two groups of Nude mice received in parallel the same doses of the same texosome preparations (|: TS/A exosomes; ?: MC38 exosomes).

No anti-tumor effect was observed in the Nude mice. T lymphocytes are therefore necessary for the texosomes' anti-tumor effects in vivo.

In the x-coordinates, we indicated the days and ranked the

average size of the tumor (mm^2).

Figure 7. Dendritic cells derived from bone marrow that have been sensitized by texosomes derived from tumor cells induce the total eradication in vivo of established solid tumors.

Five hundred thousand P815 tumor cells were injected into the right flank of DBA/2 mice 10 days prior to treatment. Treatment consisted of a single injection of texosomes (10 µg/mouse) in the same flank, but at some distance from the tumor. Another group was injected intravenously with dendritic cells (derived from bone marrow by treatment over 5 days with GM-CSF+IL4 (Mayordomo et al., 1995)); the dendritic cells were incubated earlier for 3 h with the P815 texosomes. The tumors were measured and the results analyzed as described in Figure 6. The P815 texosomes sensitized the dendritic cells in order to induce the rejection of the established tumors. The microsomes had no significant effect on tumor growth. The insert shows the percentage of tumor-free mice (in y-coordinates); the black line corresponds solely to the black-square animal groups; the x-coordinate corresponds to the days. These mice did not develop tumors following reinjection of twice the minimum tumor dose, thereby showing that they had developed anti-tumor immunity. The symbols used in the figure are as follows:

?: dendritic cells incubated with control texosomes,

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|: dendritic cells incubated with P815 texosomes,

Black triangles: Intradermal P815 texosomes,

X: untreated animals.

In x-coordinates, we indicated the days and in y-coordinates the average tumor volume.

Figures 8A, 8B, and 8C. Chemotherapeutic/cytotoxic agents and irradiation can stimulate exocytosis of tumor texosomes.

L1210 murine leukemia and the GIAM primitive human kidney cancer line were used (Figure 8C). Two million tumor cells were incubated in the presence of growing quantities of 5 Fu (for L1210) or of cis-platinum (CDDP) (for GIAM) per ml for 16 hours.

The supernatant was recovered, then underwent differential ultracentrifugation as described in Table I.

GIAM was also incubated in 100 IU/ml of IL-2, or in dexamethazone (10^{-6} M) or irradiated at 10,000 rads.

The high doses of chemotherapy and irradiation are good examples of positive regulation of texosome exocytosis in these tumor models. The results were also seen in other tumors (Figures 8A and 8B); specifically, irradiation appears to be the most powerful exocytosis stimulus.

Figure 8A corresponds to the FON melanoma described above and Figure 8B corresponds to a murine lymphoma designated as EL4 (J. Nat. Cancer Ins. (1972) 48: 265-271).

In Figure 8A, we showed the FON cells incubated in various conditions:

1) CM: basic culture medium (RPMI containing 10% fetal calf serum).
2) DXM = in the presence of dexamethazone,
3) Irradiation: irradiated at 10,000 rads,
4) Without serum,

5) IL-10 = in the presence of IL-10, at the level of 10 ng/ml.

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The above irradiations are also valid for EL4, L1210, and GIAM cells.

Figures 9A, 9B, and 9C. The membrane vesicles (dexosomes) produced by the dendritic cells sensitized to melanoma tumor antigens are effective in stimulating the specific T lymphocytes of these melanomas, and their production is regulated by the cytokines.

- LT8 (CM) corresponds to the LT8 T clones incubated in basic culture medium as defined in Figure 8A,
- DexTexNUN corresponds to the dexosome derived from dendritic cells loaded with texosomes originating from NUN tumor cells,
- DexTexFON corresponds to the dexosome derived from dendritic cells loaded with texosomes originating from FON tumor cells,
- TumFON corresponds to the FON tumor cells.

A. Proliferation Assays: the Fon texosomes (used in Figure 3) were incubated with HLA-A2 dendritic cells for 3 hours, then washed in 9% saline solution, then incubated in a pH 6.3 acid medium for 18 hours. The membrane vesicles from dendritic cells (dexosomes) are thus recovered from the culture supernatant of the HLA-A2 dendritic cells.

The membrane vesicles originating from dendritic cells loaded with texosomes (DexTex) are then incubated with the

specific MART-1 LT8 clones presented in the HLA-A2 context (the FON texosomes contain the MART-1 antigen). As a negative control, the NUN texosomes (negative HLA-A2, negative MART-1 kidney cancer line) were used. As a positive control, we used the irradiated FON tumor line (TumFON) or the anti-CD3 antibody, preabsorbed on plastic (anti-CD3Ab). Incubation of the DexTex with the LT8 lasts 48 hours, then 1 μCi of tritiated thymidine is added per 200 μl well. The LT8 lymphocyte proliferations are measured 18 hours later.

In y-coordinates, we showed the counts per minute.

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B. Same manipulations, but the IFNγ is measured in the culture supernatant at 48 hours, using ELISA. In y-coordinates, we showed the amount of IFNγ (pg/ml).

C. The dexosomes were isolated from dendritic cell supernatants after 48 hours of incubation in the presence or absence of LPS (20 μg/ml), IFNγ (100IU/ml) or IL-10 (10 μg/ml).

Figure 10. Immunoelectronic microscopy images of dexosomes. The dexosomes have a homogeneous diameter ranging from 50 to 90 nm and are strongly marked by anti-CD63 antibodies (Figure 10A). The main part of these dexosomes is also marked by anti-MHC antibodies (Page 15 [of original], Figure 10B) and anti-MCH-II antibodies (Page 15 [of original], Figure 10C). Bars: 250 nm.

Figure 11. Measurement of the levels of gamma interferon secreted by T lymphocytes incubated in the presence of peptide-loaded dexosomes or of control dexosomes. The dendritic cells (2 X 10^6/ml) were incubated for 3 to 12 hours, either in the

presence of 10 µg/ml of MART-1/MelanA$_{(27-35)}$ antigen peptide, or in the presence of 10 µg/ml of gp100 peptide$_{(280-288)}$ (control) suspended in citric acid at 3.7 pH, then the dexosomes were isolated. The cells from the LT12 clone (clone of limited HLA-A2 CTL, MART-1(27-35) specific) were then incubated (100,000 CTL per well) with increasing doses of dexosomes or of gp100 peptides (control) in 96-well plates for 5 days. The secretion of gamma interferon by the cells was then measured using ELISA (Genzyme).

Figure 12. Western Blot analysis of the markers present on the dexosomes (1.4 and 10 µg) produced by the dendritic cells derived from bone marrow: H-2K (MHC-I), I-Aα (MHC-II), CD86, CD63 TfR (transferin receptor), Clx (Calnexin), li p31 (invariable chain).

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Figure 13. Anti-tumor effect in vivo of dexosomes on a mastocytoma tumor model (P815). Dex-H2d-AEP-P815: Dexosomes from dendritic cells derived from bone marrow loaded with tumor peptide acid eluate of the P815 tumor. Dex-H2d-AEP-Spleens: Dexosomes derived from bone marrow dendritic cells loaded with spleen peptide acid eluate.

Figure 14. Anti-tumor effect in vivo of dexosomes derived from bone marrow dendritic cells loaded with tumor peptide acid eluate on a breast tumor model (TS/A). Legend: see Figure 13. (A) Experiment performed on immunocompetent mice. (B) Experiment performed on Nude mice.

Figure 15. Radioactive chromium (51Cr) release test. This test shows that the dexosomes of the invention trigger a specific

CTL response in vivo. Target cells: P815, L1210 leukemia line, YAC line insensitive to NK cells.

Figure 16. Compared efficacy of dexosomes and dendritic cells. This figure shows that the dexosomes are more powerful than the immature dendritic cells from which they are derived in eradicating established tumors in vivo. 5 million dendritic cells loaded with acid peptide eluate of the spleen (white squares) or of the P815 tumor (white triangles) were administered via intravenous or intradermic means to mice with established P815 tumors on days 8 to 10. In parallel, the supernatant of these cells was harvested after 18 h of incubation with the spleen peptides (white squares) or with the P815 tumor peptides (black triangles), ultracentrifuged, and characterized for its dexosome contents. 5 million dendritic cells yielded 5 to 10 µg of dexosomes, which enabled the immunization of 5 mice via intradermic administration in the ipsilateral flank. A single administration of dexosome was performed on days 8-10. The size of the tumors was measured twice weekly and is shown in the figure. The (*) represent 95% significant results according to Fisher's exact method, in comparison with the saline solution injections (white squares) or of pulsed dendritic cells. In the insert are shown the percentages of mice immunized against P815 showing a total absence (disappearance) of tumor during (day 21) and at the end (day 60) of the experiment, in the various groups of 5 mice.

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Figure 17. Purification of the dexosomes via electrophoresis

in fluid phase.

EXAMPLES

1. Production of Texosomes by Human and Murine Tumor Cell Lines.

This example illustrates the ability of tumor cells to produce lipid vesicles.

Murine or human tumor cells originating from leukemias or solid tumors (kidney or colon melanoma) (see Table 1) were incubated for 24 h at a density of one million cells per milliliter. The culture medium (RPMI containing 10% fetal calf serum) was then cleaned of cells by centrifugation at 300 g for 10 minutes. Cellular debris was then eliminated by two successive centrifugations of 15 min. each at 800 g (and, if necessary, a 30-minute centrifugation at 10,000 g). The texosomes were harvested by a 60-minute centrifugation at 100,000 g, then washed once in PBS under the same conditions. The protein concentration in the texosome preparations was measured using the Bradford method (BioRad Protein Assay [BioRad]).

All of the human and murine tumor lines tested (solid or hematopoietic, primary or established in culture or originating from fresh dissociated tumors) produce texosomes (Table 1). However, the production efficiency varies among various lines. The murine tumor cell lines produce between 100 and 200 micrograms of texosome proteins per 50 million cells in 24 hours. The human melanoma and nephroma lines produce between 10 and 100 micrograms of texosome proteins per 20 million cells in 24 hours.

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2. The Vesicles Produced by the Tumor Cells are of Endocytic Origin.

In order to determine whether the vesicles purified from supernatants of tumor cells lines are of endocytic origin, we performed a morphological study using electronic microscopy on one of these tumor lines, TS/A (mouse mammary carcinoma line) (Nanni P. et al. (1983) "TS/A: A New Metastasizing Cell Line Originating from a BALB/c Spontaneous Mammary Adenocarcinoma" Clin. Exp. Metastasis 1: 373-380). The tumor cells were fixed and readied for electronic microscopy as described above. The texosomes were placed directly on the grids and analyzed.

Figure 1A shows examples of intracellular compartment that are multivesicular in appearance observed in the tumor cells. These endocytic compartments have a diameter of 200-300 nm (the bar below panels A and B represents 200 nm) and are composed of an external membrane surrounding multiple internal vesicles that are 68-80 nm in diameter. The texosome preparations contain a majority population of vesicles 60-80 nm in diameter (Figure 1B), sometimes aggregated, and whose morphology is similar to the internal vesicles of the multivesicular endosomes observed inside the cells (Figure 1A). These results suggest that the texosomes are secreted into the extracellular medium after fusion of the external membrane of the endosomes with the cytoplasmic membrane. Indeed, such exocytosis profiles are observed in these cells (data not shown).

In order to determine whether the texosomes are in fact of endocytic origin, we then performed a Western Blot analysis of

the markers present - defined below - in the texosomes derived from the TS/A and P815 tumor lines (mastocytoma data by T. Boon, Ludwig Institute, Brussels, Belgium) (murine mastocytoma). In order to do this, two micrograms of texosome proteins or the cellular lysate of 200,000 TS/A cells were separated using polyacrylamide gel, then transferred onto a Nylon membrane (Amersham). The possible presence of various markers was next revealed using specific antibodies. The TS/A and P815 texosomes contain Class I MHC molecules, along with various endocytic pathway markers (transferin receptor, Lamp 1 and 2 lysosomal glycoproteins) (Figure 1A). On the other hand, a characteristic Endoplasmic Reticulum (ER) marker, calnexin, is not present in the texosome preparations, showing that the ER membranes do not contaminate the texosomes.

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<u>3. The Texosomes Produced by a Melanoma Line Contain a Cytosolic Tumor Antigen.</u>

These results show that the texosomes secreted by the tumor cells correspond to the internal membranes of the multivesicular endosomes. These intraendosomal vesicles form via invagination, then budding of the external membrane of the endosomes towards the interior of the endosome. These intraendosomal vesicles, and consequently the texosomes, should contain a cytosol fraction. This is especially important in the context of anti-tumor immunotherapy, since a certain number of tumor antigens, including MART-1 (one of the most studied antigens), are cytosolic proteins. We therefore tested the presence of MART-1 in

the texosomes.

To do this, ten micrograms of texosome proteins or the cellular lysate of 200,000 cells from a human melanoma line (M10) (T. Boon, Ludwig Institute, Brussels, Belgium) were analyzed by Western Blot, as above. The possible presence of MART-1 was revealed using a specific anti-MART-1 antibody (S. Rosenberg, NCI, Bethesda, U.S.A.). The texosomes secreted by the FON tumor line (melanoma from the FON patient coming from F. Faure, Institut Pasteur, Paris, France) contain the MART-1 tumor antigen. K (Sigma) proteinase protection experiments showed that the MART-1 epitope recognized by this monoclonal antibody is inside the texosomes (results not shown).

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This first part of the work shows that:

- tumor cells produce and secrete vesicles;
- that these vesicles are of endosomal origin and have an external membrane where various membrane molecules are found (Class I MHC, various endosome markers),
- that these vesicles contain a cytosol fraction, including cytosolic tumor antigens, such as MART-1.

These results have enabled us to verify the following biological activities of the vesicles, designated as texosomes.

<u>4. Texosomes Can Stimulate CD8 T Lymphocytes In Vitro.</u>

Since texosomes carry Class I molecules on their surface, we tested whether they are able to stimulate CD8 T lymphocytes. We used two T clones, LT8 and LT 12 provided by Faure, F., Institut Pasteur, Paris, France, recognizing a MART-1-derived peptide in

association with HLA-A2 (Dufour et al., 1997). For this purpose, since the FON tumor cells are HLA-A2, we incubated the cells of the LT8 or LT12 T clones with texosomes prepared from FON supernatants, or, as a positive control, from intact FON cells. Activation of the T lymphocytes was measured by the secretion of TNFβ. The FON texosomes induced the secretion of TNFβ by LT8 and LT8 in a dose-dependent fashion (Figure 3). The FON cells also induced a secretion of TNFβ, whereas texosomes derived from tumor cells not expressing MART-1 do not do so (Figure 3).

Therefore, HLA-A2/MART-1 peptide complexes are present on the surface of the texosomes.

Similar results were obtained in mice with T lymphocytes from the spleen of a mouse immunized with β-galactosidase (β-gal).

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Texosomes were produced from supernatants of P815 mastocytoma cells or from P815 cells expressing β-gal (lines from A. Albina, Institut Gustav Roussy, Villejuif, France), or from cells from another H2α) murine leukemia tumor (L1210) not expressing β-gal, L210. Growing concentrations (0.3 μg/ml to 20 *g/ml) of these various texosome preparations were then incubated for 4 days with spleen cells from mice immunized with β-gal expressed in a recombinant adenovirus. Only the texosomes of P815 cells (at the highest concentration of 20 μg/ml) expressing β-gal induced significant, although not strong, proliferation (see Figure 4) (measured by incorporating tritiated thymidine) of the spleen cells. These results show that the texosomes produced by

the P815 cells expressing β-gal carry β-gal-derived $H2^{\alpha}$/peptide complexes on their surface, and are able to activate murine T lymphocytes.

5. Texosomes Can Deliver Cytosolic Antigens that They Contain to Antigen Presenting Cells in Order to Be Presented to the T Lymphocytes.

For this purpose, we used LT8 and LT12 T clones that specifically recognize a MART-1-derived peptide (MART-1$_{27-25=}$AAGIGILTV, Dufour, E., et al., Diversity of the Cytotoxic Melanoma-specific Immune Response. J. Immunol. 1997, 158: 3787-3795) in association with HLA-A2. We showed that the texosomes produced by the FON human melanoma line (which is HLA-A2) contain the MART-1 tumor antigen (see Figure 2) and are able to directly activate the LT8 and LT12 clones. In order to have texosomes that also contain MART-1 but are unable to directly stimulate the LT12 and LT8 clones, we used the MZ2 melanoma line (T. Boon, Ludwig Institute, Brussels, Belgium), which is MART-1-positive but expresses an element of restriction other than HLA-A2 (in this case, HLA-A1). Indeed, the MZ2 cells, as well as the texosomes derived from these cells, do not activate the LT8 and LT12 clones (results not shown), unlike the FON cells and the texosomes derived from these cells (Figure 3). However, when these same MZ2-derived texosomes are incubated in the presence of dendritic cells expressing HLA-A2, a stimulation of the LT12 and LT8 T clones is observed, as in the case of the FON-derived texosomes (Figure 5).

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In the case of the MZ2-derived texosomes, activation of the T clones cannot be due to preexisting MART-1-derived HLA-A2/peptide complexes, since they do not express the adequate restriction element (HLA-A2). Consequently, this can only involve the antigen contained in the texosomes that was taken by the antigen presenting cells and broken down into peptides, which then were associated with the HLA-A2 molecules of the presenting cell. Therefore, the texosomes enable transfer of an antigen from a tumor cell to an antigen presenting cell. The texosomes thus play a role similar to that of "natural liposomes."

6. Texosomes Induce the Regression of Established Solid Tumors In Vivo.

Finally, since the texosomes are able to stimulate T lymphocytes in vitro and to sensitize dendritic cells for activation of specific T lymphocytes of tumors, we tested the anti-tumor activity of the texosomes in vivo.

In order to analyze such anti-tumor activity, we injected mice with twice (10^5) the minimal tumorigenic dose of tumor cells from a mammary tumor (TS/A cells of $H2^d$ haplotype, syngenic of BALB/c cells) in the flank. After 3 or 4 days, the animals with established tumors were injected twice (day 3 and 4) with texosomes prepared from TS/A cell supernatants or, as a negative control, with texosomes of MC38 cells (S. Rosenberg, NCI, Bethesda, U.S.A.) ($H2^b$ haplotype tumor cell) or a similar volume of PBS. The average tumor size in the group of mice inoculated with TS/A texosome preparations was greatly diminished in comparison with the control group mice. This anti-tumor effect is

T-cell-dependent, because Nude mice (mutant mice devoid of T lymphocytes) bearing the tumor and inoculated in similar fashion with texosome preparations do not show any diminution in the tumor mass (Figure 6B).

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In this same series of experiments, we observed an anti-tumor effect of the texosomes prepared from P815 mastocytoma cells of $H2^d$ haplotype, which leads us to believe that these two tumors express common antigens. Similar results were obtained with another, highly immunogenic model, P815 mastocytoma (syngenic of DBA/2 mice and of $H2^d$ haplotype). In this model, we showed that texosomes injected intradermally into the flank of a mouse bearing an established tumor (P815) on the 10th day (tumor measuring 80-100 mm^2) are able to lead to eradication of the tumor in over 60% of cases. Moreover, these mice show long-term anti-tumor immunity (results not shown). In this series of experiments, we observed anti-tumor effects of texosomes prepared from L1210 lymphocytes isolated from a murine leukemia of $H2^d$ haplotype and from TS/A cells, which indicates that common epitopes also exist among these three tumors (between P815 and TS/A, mutated p53 is common to the two tumors).

Two mechanisms could be the basis for the anti-tumor effect of the texosomes.

First of all, once they are injected, the texosomes could directly activate the host's T lymphocytes that are specific to the tumor. In this way, a clonal expansion of tumor-specific T lymphocytes or a "priming" of T cells could take place. A second

hypothesis implies a direct interaction of the injected texosomes with the host's dendritic cells. This could then stimulate the anti-tumor response. In order to test this second hypothesis, we followed the growth of tumors in mice that were intravenously injected with bone-marrow-derived dendritic cells loaded with tumor cell texosomes. DBA/2 mice (IFFA CREDO, Orléans, France) were thus injected with twice (50×10^5) the minimal tumorigenic dose of P815 mastocytoma cells. Ten days thereafter, each animal was injected with 5×10^5 loaded dendritic cells and, sensitized following this, with 9 µg of texosomes. The average tumor size in these conditions decreases significantly in comparison with groups of mice injected with PBS or with dendritic cells loaded with texosomes from cells of an MC38 control tumor. Indeed, over 60% of the treated animals no longer show evidence of a tumor at the end of the experiment. Additionally, long-term recidivism has not been observed (in 80% to 100% of cases). It is interesting to note that such a suboptimal dose of texosomes injected intradermally has no effect, which suggests that the dendritic cells can prepare dexosomes containing the tumor antigens much more efficiently that the dendritic cells of the derma or Langerhans cells. We obtained similar results with the TS/A breast tumor cell model.

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The results obtained in the context of the invention show that texosomes effectively sensitize dendritic cells. Thus sensitized, these cells are able to induce powerful anti-tumor responses in vivo for various tumors. These results suggest that

the anti-tumor effects observed after the direct inoculation of texosomes in vivo are due to the sensitization of the host's dendritic cells. Remarkably, the effect is observed after a single injection of sensitized dendritic cells. The majority of treated mice show total tumor regression or prolonged survival (60 days versus 20 days in the control group) due to tumor regression.

The method for sensitizing the presenting cells of the invention has various advantages over the methods of the prior art:

i) it does not require advance knowledge of tumor antigens: this is especially important because tumor antigens are not known in the vast majority of tumors;

ii) the process of the invention may be applied to any tumor that produces texosomes; out of over 15 lines of tumor cells tested to date, only one does not produce texosomes (one of the six melanoma lines tested).

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iii) this method does not depend on the haplotype of the patient's MHC and of the tumor cells, since the tumor antigens present in the texosomes are re-readied and presented to the T lymphocytes by molecules of the MHC of the patient's antigen presenting cells;

iv) the process of the invention may, in theory, be effective on tumors of various origins, since tumor antigens exist that are common not only to a particular type of tumor (MART-A, for example, in melanoma), but

there are also antigens that are common to completely different tumors (such as the molecules involved in tumorigenesis, p53, for example);

v) the use of texosomes may also prove effective in treating tumors that express low levels of Class I MHC molecules, or that do not express any at all (these tumors represent 40-50% of human metastatic cancers). Indeed, texosomes enable the transfer of intact antigens between tumor cells and dendritic cells; these antigens are then presented to the T lymphocytes by the MHC molecules of the dendritic cell. Preliminary results show that texosomes induce rejection of murine tumors expressing low levels of Class I MHC molecules (such as MCA101, S. Rosenberg, NCI, Bethesda, U.S.A.). This is probably due to the fact that the levels of expression of MHC molecules necessary for inducing an effective immune response are much higher than those needed during the effecting phase (cellular cytotoxicity);

vi) texosomes alone constitute in and of themselves, through their immunogenic potential, a novel and effective modality for prophylactic or therapeutic vaccination.

7. Dendritic Cells Produce Immunogenic Membrane Vesicles (Dexosomes)

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This example demonstrates that dendritic cells produce

membrane vesicles and that these vesicles constitute powerful immunogenic vesicles for anti-tumor immunization. These vesicles are especially advantageous because they make it possible to avoid the stage of in vivo injection of the whole dendritic cells from which they originate.

Dendritic cellular therapy does not offer the certainty of the stable phenotype of the injected cell, nor the homogeneity of the cellular compositions used. By administering a stable product secreted by these cells, that is, the membrane vesicles described above, the tumor-bearing host is offered guaranteed efficacy and immunizing potential.

In this example, dendritic cells derived from bone marrow by treatment over 5 days with GM-CSF+IL-4 (Mayordomo et al., 1995) were incubated for 3 hours with exosomes from tumor cells. Thus sensitized, the cells were then cultivated in an acid medium for 18 hours (in order to stimulate the production of vesicles), then vesicles were observed and harvested according to the methodology described in Example 1. In order to determine their immunogenic potential, these vesicles were then incubated in vitro with specific cytotoxic T lymphocytes of the MART-1 antigen. The results presented in Figure 9 show that 0.6 μg of these membrane vesicles, called DexTexFON (that is, dexosome originating from dendritic cells incubated with texosomes originating from FON tumor cells), directly stimulates the proliferation and secretion of IFNγ of MART-1-specific LT8 clones; this is what 0.6 μg of dexosomes originating from dendritic cells incubated with NUN tumor cell texosomes (DexTexNUN: NUN being a kidney tumor that is

HLAA2 negative, MART-1 negative) is unable to do. These results therefore show (i) that dendritic cells produce membrane vesicles and that these membrane vesicles constitute a powerful immunogene.

Moreover, the results presented in Figure 9C show that, unexpectedly, the production of dexosomes for the dendritic cells is a regulated phenomenon, which can be stimulated in the presence of certain cytokines such as IFN-γ and IL-10. Thus, the results presented show that IFN-γ or Il-10 significantly increase (by a factor of 5, approximately) the production of dexosomes. Comparable results were observed with IL-12.

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8. Characterization of the Membrane Vesicles Produced by the Dendritic Cells.

The ability of dendritic cells to produce membrane vesicles was initially confirmed on dendritic cells produced from human monocyte precursors and on the D1 line of murine dendritic cells.

The D1 cell line and the maturation conditions of this line were described by Winzler et al. (J. Exp. Med. 185 (1997) 317).

The dendritic cells derived from human monocyte precursors were obtained from the adherent fraction of mononucleated cells, sampled from healthy subjects, incubated for 7-8 days in AIMV medium containing L-Glu, antibiotics, 1000 IU/ml of rhGM-CSF and rhIL4 (Schering-Plough, Kenilworth, NJ, USA). After 8 days of culturing, the weakly adherent cells and the cells in suspension present a morphology that is typical of dendritic cells, expressing high levels of Class I and II MHC molecules, as well

as CD40 and CD86. The major part of these cells is positive for CD1a and CD11b and negative for CD2, CD3, CD14, CD19, and CD83.

Microscopic analyses of these cells showed the presence of membrane vesicles rich in Class I and II MHC molecules. These vesicles were isolated by centrifugations and analyzed using immuno-electronic microscopy. More specifically, the culture supernatants of the dendritic cells were harvested, centrifuged at 300 g for 20 minutes then at 10,000 g for 30 minutes at 4 degrees C, in order to eliminate the cells and the cellular debris. The dexosomes were then isolated using centrifugation at 100,000 g for 1 h at 4 degrees C, following by washing in PBS under the same conditions (centrifugation at 100,000 g for 1 h at 4 degrees C). The protein concentration in the dexosome preparations was measured using the Bradford method (BioRad Protein Assay [BioRad]).

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The results obtained show (Figure 10) a homogeneous population of vesicles having a diameter ranging from 60 to 90 nm approximately. Additionally, over 95% of the dexosomes are marked by anti-CD63, anti-CD82, anti-MHC-I and anti-MHC-II antibodies.

These results confirm that dendritic cells produce membrane vesicles presenting antigen presenting molecules as well as lymphocyte costimulation molecules.

9. Dexosomes Present the Antigens in a Restricted MHC-I Context.

One of the advantageous characteristics of the dexosomes resides in the presence of Class I MHC molecules. These molecules

are necessary for generating an effective cellular response, especially for activation and expansion of CTL cells. The dexosomes' ability to stimulate CD8+ lymphocytes and the specific character of the obtained lymphocytes were therefore tested.

To do this, the dendritic cells obtained from human monocyte precursors (from HLA-A2 subjects) were initially sensitized to a specific antigen by "peptide pulsing." Towards this end, the cells (2×10^6/ml) were incubated from 3 to 12 hours, either in the presence of 10 µg/ml of the MART-1/MelanA$_{(27-35)}$ antigen peptide, or in the presence of 10 µg/ml of gp100$_{(280-288)}$ peptide (control) suspended in pH 3.7 citric acid. After this sensitization stage, the dexosomes were isolated as described in Example 1. The cells from the LT12 clone (clone of restricted HLA-A2 CTL, MART-1(27-35) specific) were then incubated (100,000 CTL per well) with increasing doses of dexosomes or of gp100 peptides (control) in 96-well plates for 5 days. The secretion of gamma interferon by the cells was then measured by ELISA (Genzyme).

As is shown in Figure 11, the dexosomes carrying the MART-1 peptide are able to stimulate the production of gamma interferon by the LT12 clone in a dose-dependent fashion. However the dexosomes produced from dendritic cells pulsed with the control gp100 peptide do not have any stimulating effect on this clone.

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These results confirm that the MHC-I molecules expressed by the dexosomes of the invention are functional.

10. Dexosomes Block Tumor Growth In Vivo.

This example demonstrates the ability of the dexosomes of the invention to induce an immune response in vivo and more specifically to induce the proliferation of specific T cells of a tumor.

Bone-marrow-derived dendritic cells were loaded with an acid tumor eluate including various tumor antigen peptides. The technology for preparing and sensitizing dendritic cells was described by Zitvogel et al. (1996). Figure 12 presents the markers expressed by the dexosomes produced by these dendritic cells. As was indicated previously, this figure shows the abundant presence of Class I and Class II MHC molecules, as well as CD86 markers and the transferin receptor. However, although they appear in the cellular lysates, the H2-M, li, and calnexin markers are undetectable in the exosomal preparations.

Two experimental tumor models were selected in order to test the anti-tumor properties in vivo of the dexosomes of the invention. The first model, P815, is an aggressive mastocytoma syngeneic of DBA/2 ($H2^d$) for which very few effective immunotherapies have been reported in established tumors on day 10. The TS-A model is a spontaneous, weakly-immunogenic mammary carcinoma that expresses lower levels of Class I MHC molecules, and is syngeneic of BALB/c ($H2^d$). The tumor peptides of the P815 or TS/A tumors, eluated by acid treatment, were loaded onto the bone-marrow-derived syngeneic dendritic cells as described above. The dexosomes were then prepared from supernatants of these dendritic cells and used for in vivo immunization.

Mice and Tumor Cell Lines.

Female DBA/2J ($H2^d$) and BALB/c ($H2^d$) mice, six to eight weeks of age, were purchased at Laboratoire Iffa Credo, Lyon, France, and kept in pathogen-free conditions. The nude mice were kept in a protected microenvironment. The P815 cells were provided by T. Boon (Ludwig Institute, Belgium). The TS/A model was provided by Guido Forni (Immunogenetic and Histocompatibility Center, Turin, Italy). All of the tumor lines were kept in RPMI 1640 medium supplemented by 10% endotoxin-free fetal calf serum (Gibco BRL), 2mM of L-Glutamine, 100 units/ml of penicillin, 100 mg/ml of streptomycin, essential amino acids, and pyruvate. This medium is also designated in the following as: CM medium.

Protocol and Results

Twice the minimal tumorigenic dose of tumor cells (5×10^5 P815, 10^5 TS/A) were intradermally inoculated into the upper zone of the right flank of the DBA/2 and BALB/c mice, respectively. The animals presenting TS/A tumors established for three to four days or P815 tumors established for six to ten days were then immunized by a single intradermal injection of 3 to 5 µg of dexosomes in the lower part of the ipsilateral flank. These procedures were carried out similarly both in the immunocompetent animals and in the nude mice. A single therapeutic injection was performed on each mouse. The size of the tumors was checked twice weekly and the mice were killed when they bore ulcerated or overly large tumors. Each experiment series was performed two to three times, using groups of five mice for each individual treatment. The results obtained are presented in Figure 13. As

Figure 13B shows, the treatment of established P815 tumors on day 10 (having a size ranging from 50 to 90 mm^2) was performed by a single intradermal administration of 3 to 5 µg of dexosomes per mouse. In one week, tumor growth had stopped in the groups that had received the dexosomes derived from dendritic cells loaded with the autologous tumor peptide, and in 40 to 60% of the mice, the tumors had entirely disappeared on day 60.

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Additionally, these animals present a sustainable immune response and rejected a supplementary P815 injection that is normally lethal to untreated mice. However, these mice are not protected against an injection of the L1210 syngeneic leukemia clone, which clearly shows the immunospecific character of the obtained effect. Finally, the groups of mice immunized with the control dexosomes (loaded with mouse spleen peptides) show no anti-tumor effect, nor do the untreated mice. These results therefore show that the dexosomes loaded with tumor peptides according to the invention are able to induce tumor regression in vivo.

Similar anti-tumor effects were obtained on the TS/A tumor model including tumors established on days 3/4. In this series of experiments, all of the mice showed statistically significant delays in tumor growth, which prolonged their survival (Figure 14). This anti-tumor effect was not observed in the Nu/Nu athymic mice as indicated in Figure 14B, which shows that the presence of T cells is necessary for the expression of the anti-tumor effect of the dexosomes of the invention.

Moreover, the following experiment shows that the dexosomes directly stimulate a specific CTL response in the animals presenting the P815 tumor. The mouse splenocytes that rejected the P815 tumors after immunization with the dexosomes were harvested on day 90 and cultivated for five days in the presence of irradiated P815 cells expressing the B7.1 antigen in order to increase the frequency of specific precursors. These effector cells were tested in a chromium release test against (i) the autologous P815 ($H2^d$) tumor cells, (ii) the non-related L1210 cells and (iii) the YAC cells. A significant specific cytolytic activity was observed against the P815 cells in the mouse splenocytes immunized with the dexosomes (Figure 15). Interestingly, none of the mouse spleens spontaneously rejecting the P815 tumor or presenting P815 tumors presents this cytolytic activity under the same conditions. These results show that a single injection of dexosomes according to the invention, sensitized with an antigen or corresponding antigen peptides, is able to effectively trigger a specific CTL anti-tumor response in vivo.

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In order to determine whether the immune and anti-tumor response induced by the dexosomes is restricted to the MHCs and is not simply caused by a direct effect of the tumor peptides, dendritic cells derived from $H2^d$ (DB/A) or $H2^b$ (C57BL/6) mice were loaded in parallel with eluated tumor peptides of the P815 tumor. The dexosomes produced by these dendritic mouse cells were then isolated and used separately for direct intradermal

injections into the DBA/2 mice having established P815 tumors on days 6-10.

As Figure 13B shows, only the dexosomes carrying the syngeneic tumor peptides are effective anti-tumor vaccines (inducing disappearance of the tumor in 60% of mice) whereas the dexosomes of the allogenic dendritic cells induce practically no anti-tumor effect. These results indicate that the dexosomes according to the invention induce an anti-tumor response in vivo that is restricted to MHC.

Experiments similar to those described above were performed by proceeding with injections that were not intradermal, but intravenous. The results obtained are presented in Figure 16. First of all, they show tumor regression consecutive to intravenous injection of dexosomes. Moreover, these results show that dexosomes are more powerful than the dendritic cells from which they are derived in eradicating tumors in vivo. These results therefore illustrate the remarkable and unexpected properties of dexosomes.

The results presented above show that immature human or murine dendritic cells secrete dexosomes, that these dexosomes present not only Class II MHC molecules, but also Class I MHC molecules, as well as costimulating molecules, and lastly that these dexosomes are immunogenic and induce tumor regression in vivo.

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The dexosomes may be obtained in relatively large quantities (1 µg per million dendritic cells per eighteen hours, according

to the Bradford test) from media of dendritic cell cultures (bone-marrow-derived dendritic cells in the presence of GM-CSF + IL4, D1 dendritic cell lines or dendritic cells derived from human monocyte precursors, isolated from mononucleated peripheral blood cells). The dexosomes were morphologically and biochemically characterized. The membrane vesicles analyzed by immunoelectric microscopy represent a homogeneous population of vesicles having a diameter of approximately 60 to 90 nanometers. The dexosomal preparations are apparently free from retroviruses, plasmic membranes, microsomal constituents, and apoptotic bodies. The dexosomes abundantly overexpress Class I and II MHC molecules, CD63 molecules, and CD86 molecules in comparison to plasmic membranes. No endoplasmic reticulum-derived compartment was detected in the dexosomes by Western Blot using anticalnexin antibodies. Programmed cell death was not seen in these cultures under various conditions. Interestingly, production of these vesicles appears to be a regulated phenomenon. It appears that the quantity of vesicles can be decreased by inducing maturation of the dendritic cells, as determined by Bradford test, Western Blot, and immunoelectronic microscopy. Moreover, the level of secretion of these vesicles can be significantly improved by lowering the pH of the culture medium, or by incubating the cells in the presence of certain cytokines, or by treating the dendritic cells by irradiation. This phenomenon is especially unexpected because immature dendritic cells are generally considered to have low antigen presentation potential and therefore a low level of immunological activity. The results

presented show that dendritic cells at this immature stage have the property of producing dexosomes efficaciously. The results presented show, finally, that these dexosomes are able to trigger a response by efficacious T cells both in vitro and in vivo and that they are also able to induce tumor regression in vivo. Therefore, these vesicles constitute especially attractive candidates for immunotherapy in non-cellular systems.

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11. Purification of Exosomes by Electrophoresis in Fluid Phase.

This example describes the use of an original method for purifying exosomes based on electrophoresis in fluid phase.

Electrophoresis in fluid phase is a preparative method for separating biological compounds according to their load. This method was used to separate proteins by isoelectrofocalization. This method may offer the following advantages:

- it is a preparative method that enables a continuous injection of material, and therefore the purification of large quantities of vesicles.
- This method enables purification of dexosomes in one or two stages, potentially eliminating the centrifugation stages.

In order to determine whether this method is applicable to exosome purification, we performed the following experiment:

A preparation of dexosomes isolated from a supernatant of murine dendritic cells by differential ultracentrifugation was injected into the electrophoresis during the fluid phase, in the usual conditions described by Amigorena et al. (Nature, 369

(1994), 113). 40 fractions were harvested and the protein concentration in each of these fractions was determined by the Bradford test (BioRad), in order to detect the presence of dexosomes. As Figure 17 shows, 90% of the dexosomes were found concentrated in four electrophoresis in fluid phase fractions. The migration of the dexosomes into a narrow peak according to this technology demonstrates the feasibility of electrophoresis as a method for isolating dexosomes.

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References

[References in English; please refer to original document]

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[References in English; please refer to original document]

Table 1. Texosome Production by Murine and Human Tumor Cell Lines.

LIGNÉES DE CELLULES TUMORALES 1	TEXOSOMES (µG/2x10^7 CELLULES/18 H) 2
Murines	
MCA101	172
P815	163
MC38	120
L1210	150
TS/A	160
Humaines (mélanomes) 3	
VIO*	80
FON	90
MZ2-2	18
Humaines (néphromes) 4	
RCC NUN*	120
RCC JOUA*	18
RCC MEG*	10
RCC GIAM*	100

[Key: 1. Tumor Cell Lines; 2. Cells; 3. Human (melanomas); 4. Human (nephromas)]

CLAIMS

1. Vesicle derived from a tumor cell characterized in that:

- it is removed from its natural environment, and
- it includes a lipid bilayer that surrounds a cytosolic fraction.

2. Vesicle according to Claim 1, characterized in that it presents on its surface Class I and/or Class II major histocompatibility complex (MHC) molecules.

3. Vesicle according to Claim 1 or 2, characterized in that it also presents on its surface adhesion molecules and/or lymphocyte costimulation molecules.

4. Vesicle according to one of Claims 1 through 3, characterized in that it also presents on its surface antigen peptides, possibly associated with the Class I/II MHC molecules.

5. Vesicle according to one of Claims 1 through 4, characterized in that it contains tumor antigen molecules and/or immunomodulators and/or chemoattractors and/or hormones and/or nucleic acids in its cytosolic fraction.

6. Vesicle derived from a tumor cell characterized in that it has a diameter ranging from 60 to 90 nm, in that it has on its surface antigen peptides that are characteristic of tumors in association with Class I and/or Class II major histocompatibility complex (MHC) molecules, and in that it has on its surface lymphocytic costimulation molecules.

7. Vesicle according to Claim 6, characterized in that it contains the HSP70 protein.

8. Vesicle according to Claim 6 or 7, characterized in that it is devoid of the gp96 protein.

9. Vesicle according to any of the preceding claims, characterized in that it also contains a heterologous nucleic acid.

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10. Vesicle preparation process according to one of Claims 1 through 9 (texosomes) including a first stage for making available a biological sample and a second stage for isolating texosomes from the sample.

11. Process according to Claim 10, characterized in that the biological sample is constituted of membrane fractions, culture supernatants, or tumor cell lysate, or of fresh tumor suspensions.

12. Process according to Claim 10 or 11, characterized in that the biological sample is treated with one or several texosome production-stimulating agents.

13. Process according to Claim 10, characterized in that the isolation is performed by centrifugation, electrophoresis, chromatography, and/or nanofiltration.

14. Antigen presenting cell loaded in vesicle (texosome) according to one of the Claims 1 through 9.

15. Process for preparing an antigen presenting cell according to Claim 14 including the stages for incubation of antigen presenting cells in the presence of one or several texosomes according to one of Claims 1 through 7, and for recovering the loaded antigen presenting cells thus obtained.

16. Membrane vesicle removed from its natural environment, secreted by vesicle-loaded antigen presenting cells according to one of Claims 1 through 9.

17. Membrane vesicle (designated "dexosome"), characterized in that it derives from a dendritic cell, it includes one or several Class I major histocompatibility complex molecules, and it includes one or several Class II major histocompatibility complex molecules.

18. Vesicle according to Claim 17, characterized in that it also includes one or several CD63 molecules.

/64

19. Vesicle according to Claim 17 or 18, characterized in that it also includes one or several CD82 and/or CD86 molecules, preferably at least CD86.

20. Vesicle according to one of Claims 17 through 19, characterized by a diameter ranging from 60 to 90 nm.

21. Vesicle according to one of Claims 17 through 20, characterized in that it also includes one or several antigen peptides.

22. Vesicle according to one of Claims 17 through 21, characterized in that it derives from an immature dendritic cell.

23. Vesicle according to one of Claims 17 through 22, characterized in that it derives from a dendritic cell that carries one or several antigen peptides.

24. Vesicle according to Claim 23, characterized in that it derives from a dendritic cell incubated with a vesicle according to Claim 1.

25. Vesicle according to Claim 23, characterized in that it derives from an immortalized dendritic cell.

26. Vesicle preparation process according to one of Claims 17 through 25, including a first stage for obtaining dendritic cells or of a cell culture including dendritic cells, a second optional stage, during which the cells can be sensitized to antigens of interest, and a third stage including the production of vesicles from these cell cultures.

27. Process according to Claim 26, characterized in that the first stage includes obtaining dendritic cells from monocytes precursors or from bone marrow.

28. Process according to Claims 26 or 27, characterized in that the first stage includes obtaining immature dendritic cells, preferably human ones.

29. Process according to one of Claims 26 to 28, characterized in that the sensitization stage is carried out by placing the dendritic cells in contact with peptides, antigens, cells or membranes or vesicles expressing antigens or antigen peptides, liposomes or nucleic acids, possibly incorporated into chemical or viral vectors.

/65

30. Process according to one of Claims 26 through 29, characterized in that the vesicle preparation stage includes a first, optional cell treatment stage, followed by a second vesicle isolation stage.

31. Process according to Claim 30, characterized in that the dendritic cells are treated by culturing in the presence of

cytokines encouraging the immature state, by irradiation, or by lowering the culture's pH, or by combining these various treatment types.

32. Process according to Claim 30 characterized in that the isolation of the vesicles is performed according to Claim 31.

33. Process for preparing cell-derived membrane vesicles including at least one separation stage using electrophoresis, chromatography, or nanofiltration.

34. Use:

- of texosomes according to one of Claims 1 through 9, or
- of antigen presenting cells according to Claim 14, or
- of dexosomes according to one of Claims 16 through 25, for the stimulation and possible amplification in vitro of specific T lymphocytes of antigens contained in the texosomes, antigen presenting cells, or dexosomes, or of B lymphocytes, and specifically for the stimulation and amplification in vitro of T lymphocytes.

35. Use of texosomes according to one of Claims 1 through 9, or of antigen presenting cells according to Claim 14, or of dexosomes according to one of Claims 16 through 25, for the selection ex vivo of a repertoire of T lymphocytes that are likely to recognize specific antigens contained in the texosomes, antigen presenting cells, or dexosomes.

36. Drug including as an active ingredient one or several texosomes according to one of Claims 1 through 9, antigen presenting cells according to Claim 14, and/or dexosomes according to one of Claims 16 through 25, in association with a

pharmaceutically-acceptable vehicle.

/66

37. Drug according to Claim 36 for treating cancer and infectious and parasitic diseases.

38. Drug according to Claim 35 or 36 characterized in that it also contains a stabilizing agent.

39. Association of texosomes and/or dexosomes and an immunostimulating adjuvant, for the purpose of simultaneous, separate, or at various time intervals.

40. Use of a dexosome according to one of Claims 16 through 25 for the preparation of a pharmaceutical composition to be used for treatment of cancer or infectious and parasitic diseases.

41. Use of gamma interferon, interleukin 10 and/or interleukin 12 for dexosome production.

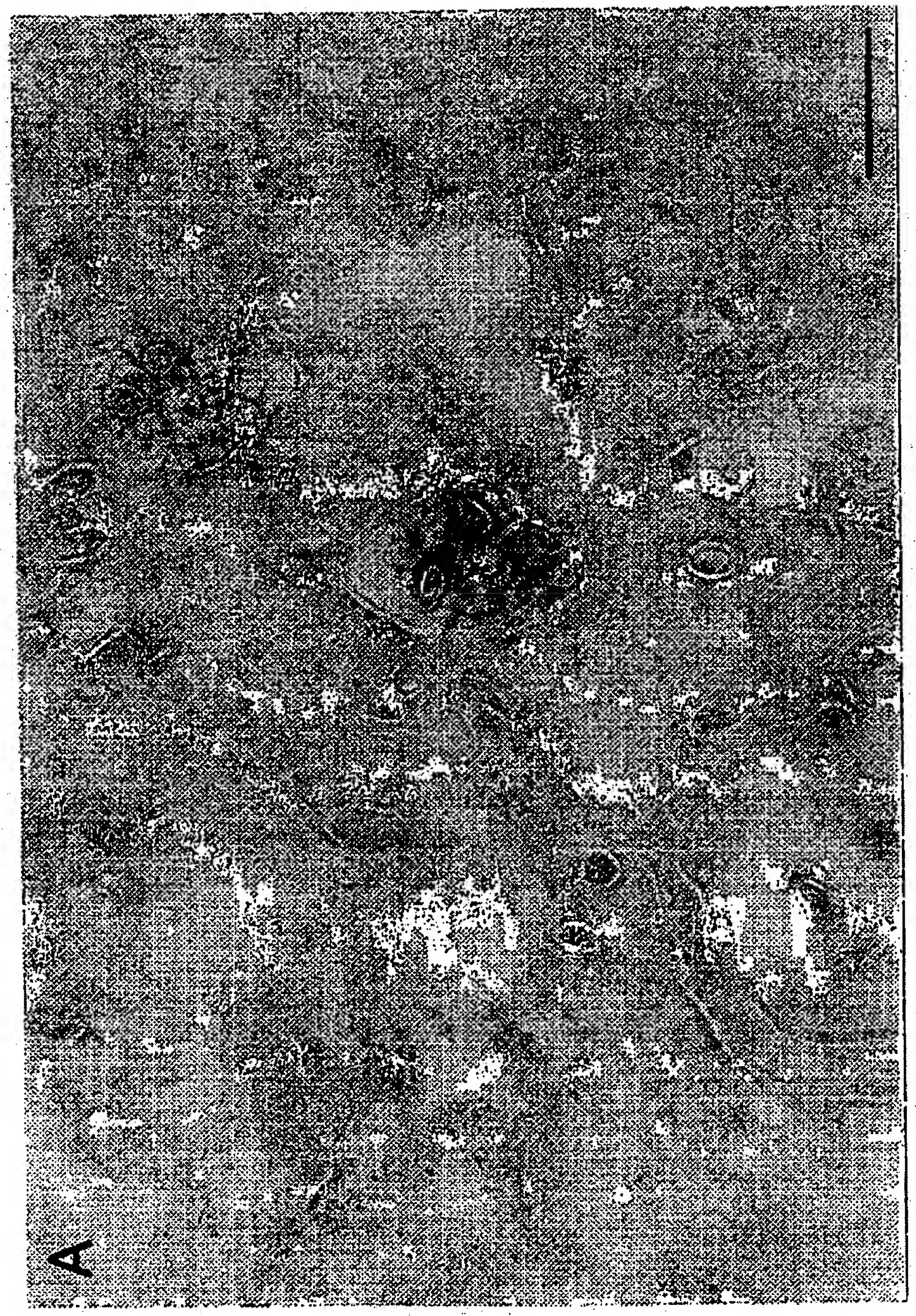

Figure 1A.

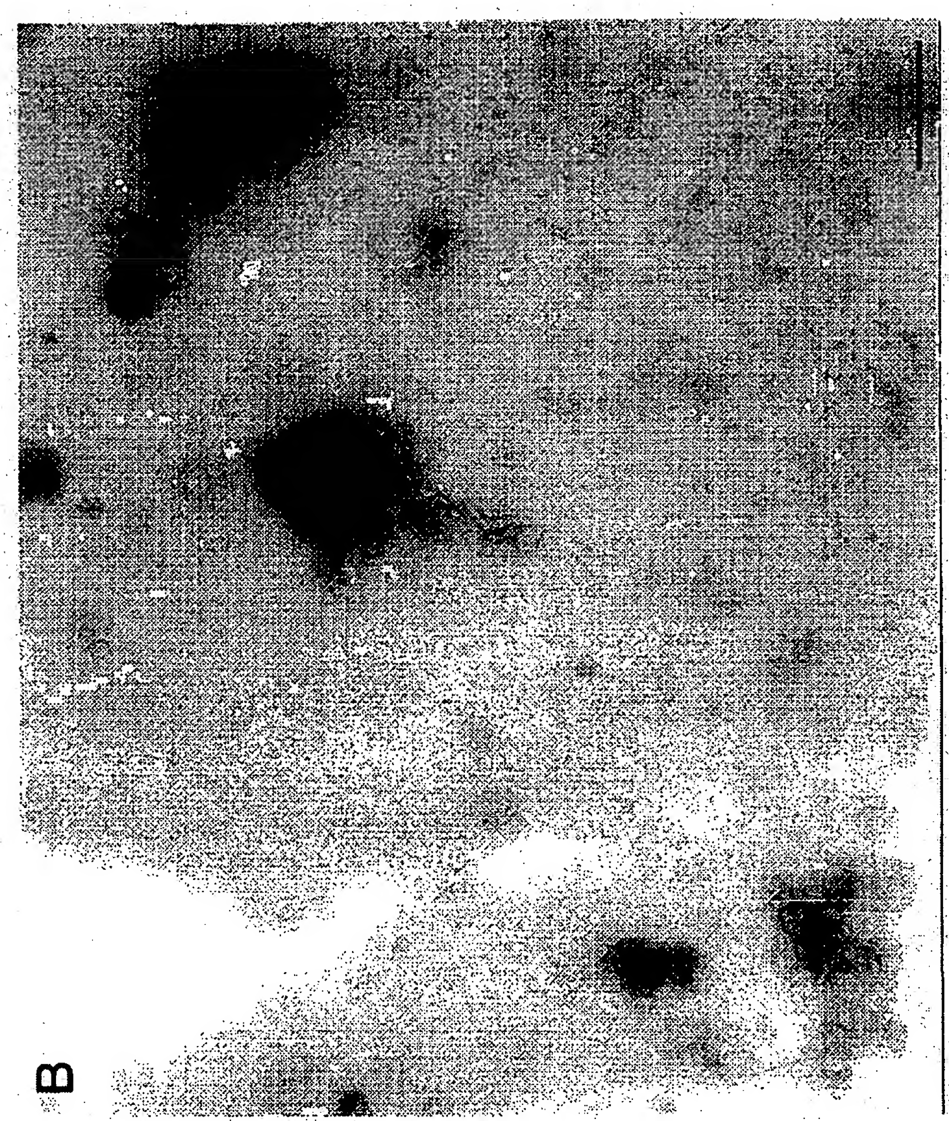

Figure 1B.

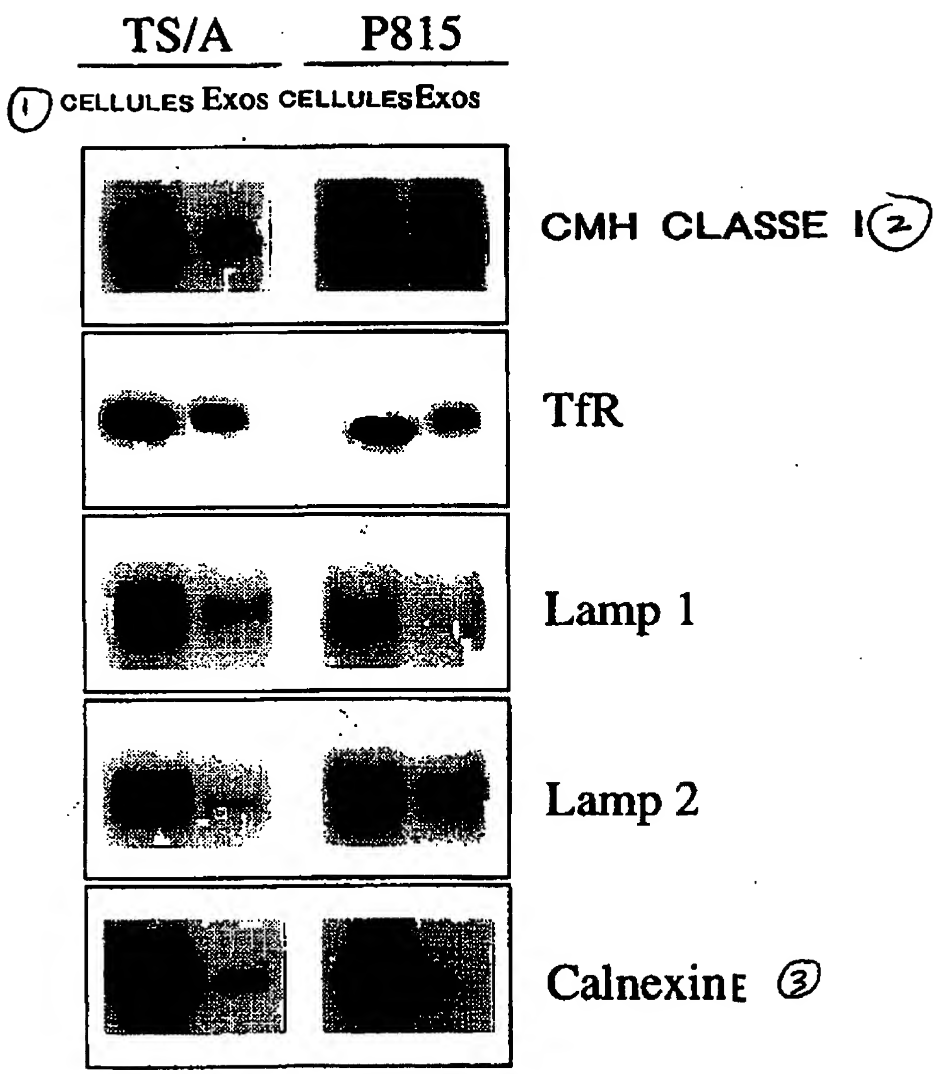

Figure 2A.

[Key: 1. Exos cells; 2. Class I MHC; 3. Calnexin]

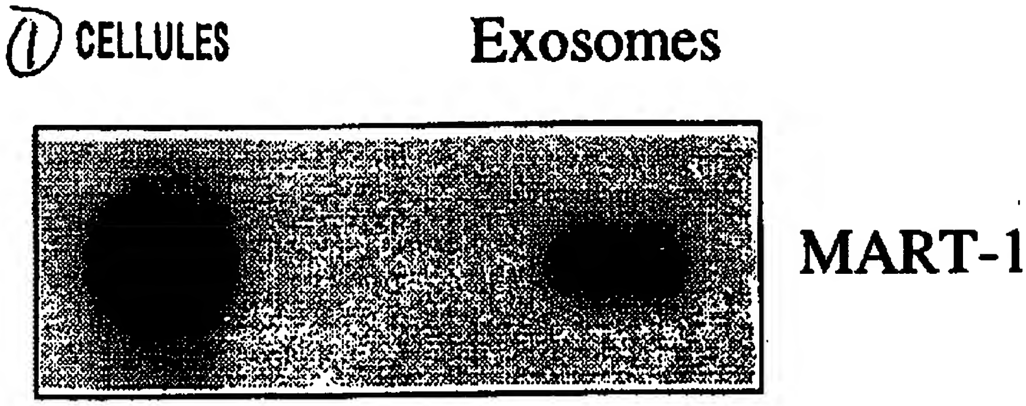

Figure 2B.

[Key: 1. Cells]

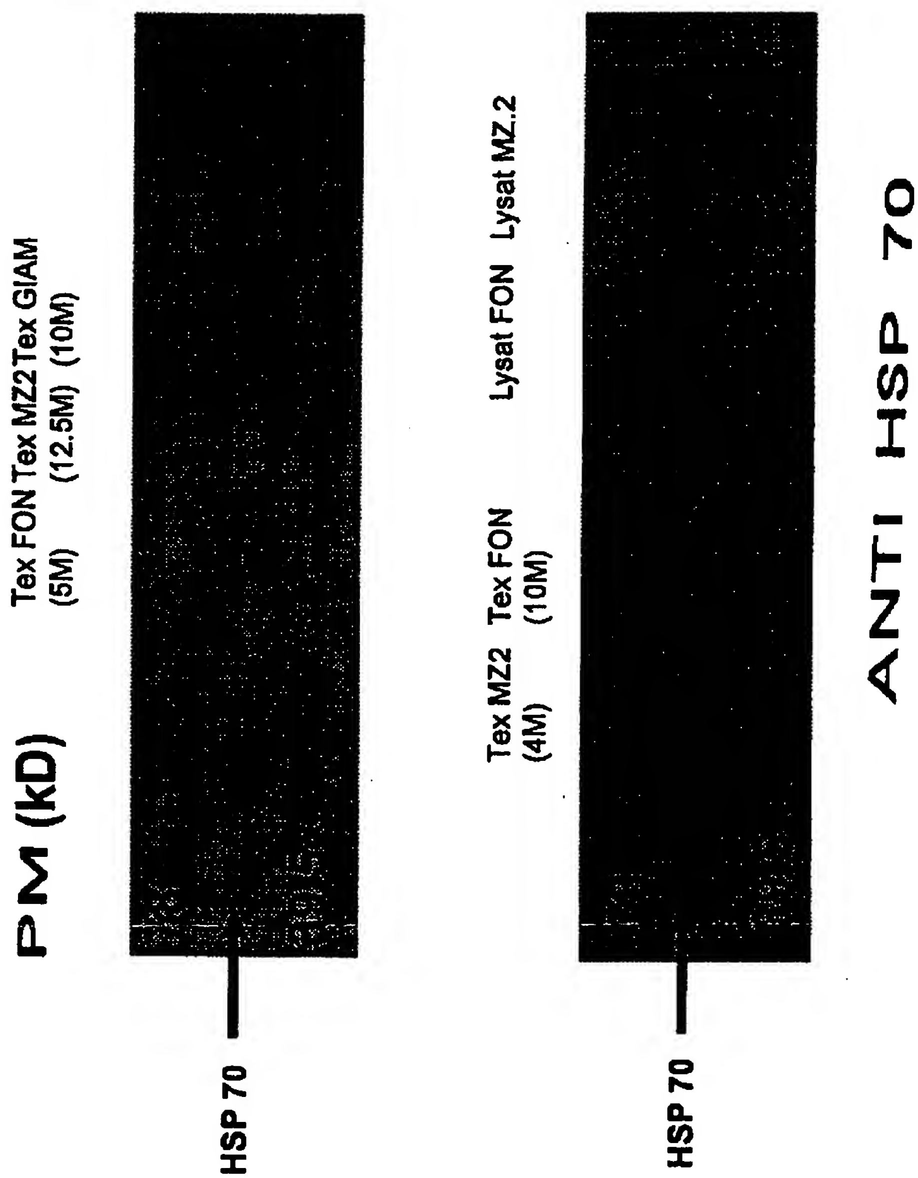

FIGURE 2C

Figure 2C.

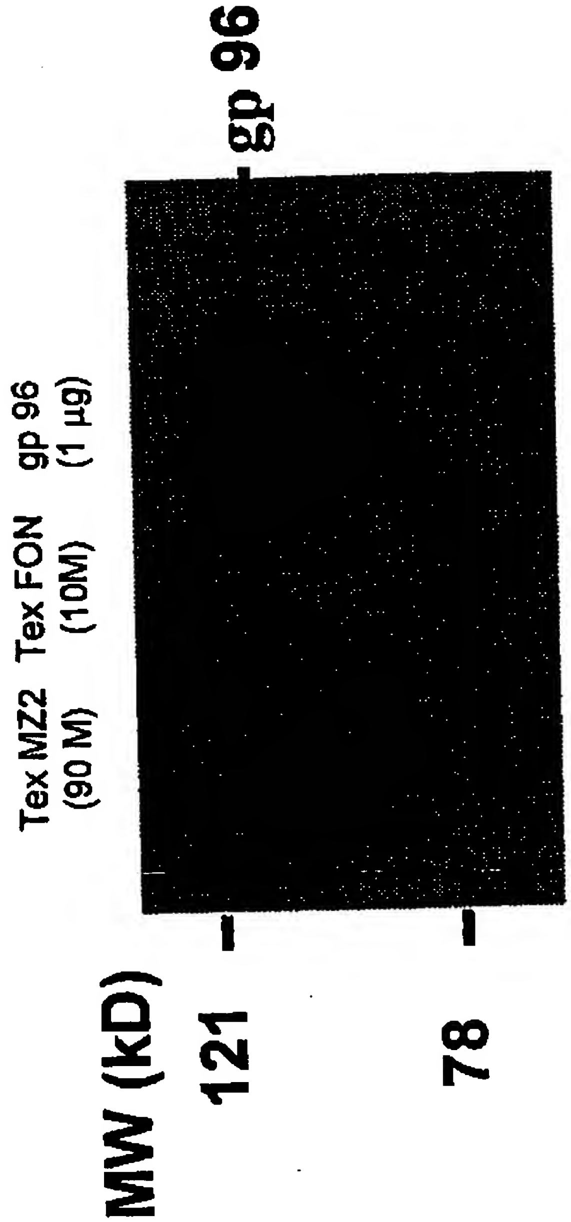

Figure 2D.

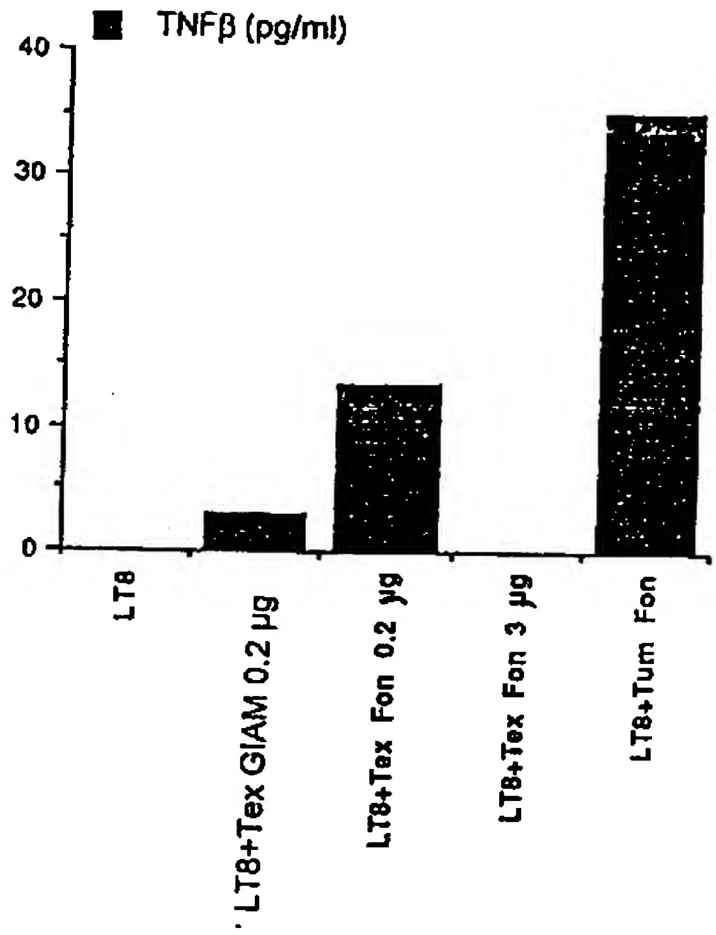
TNFβ (pg/ml)
40
30
20
10
0
LT8
LT8+Tex GIAM 0.2 µg
LT8+Tex Fon 0.2 µg
LT8+Tex Fon 3 µg
LT8+Tum Fon

Figure 3.

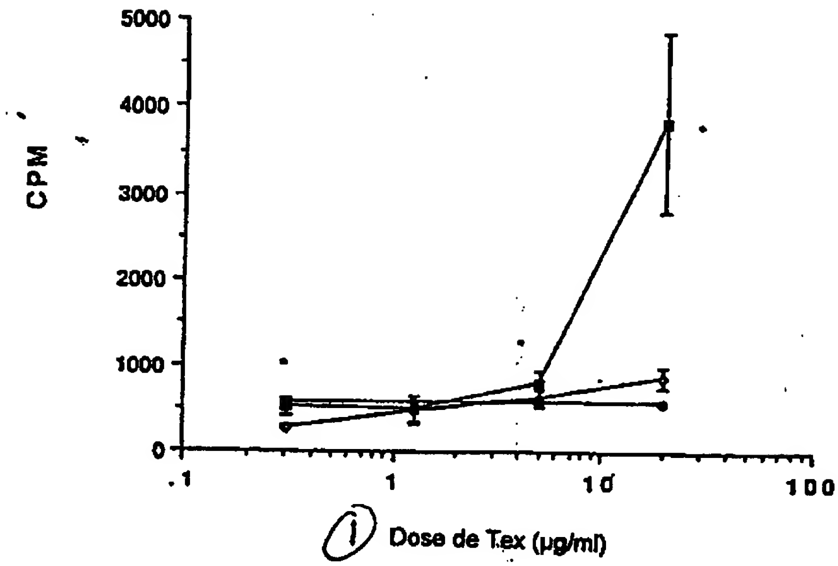

Figure 4.

[Key: 1. Dose of Tex]

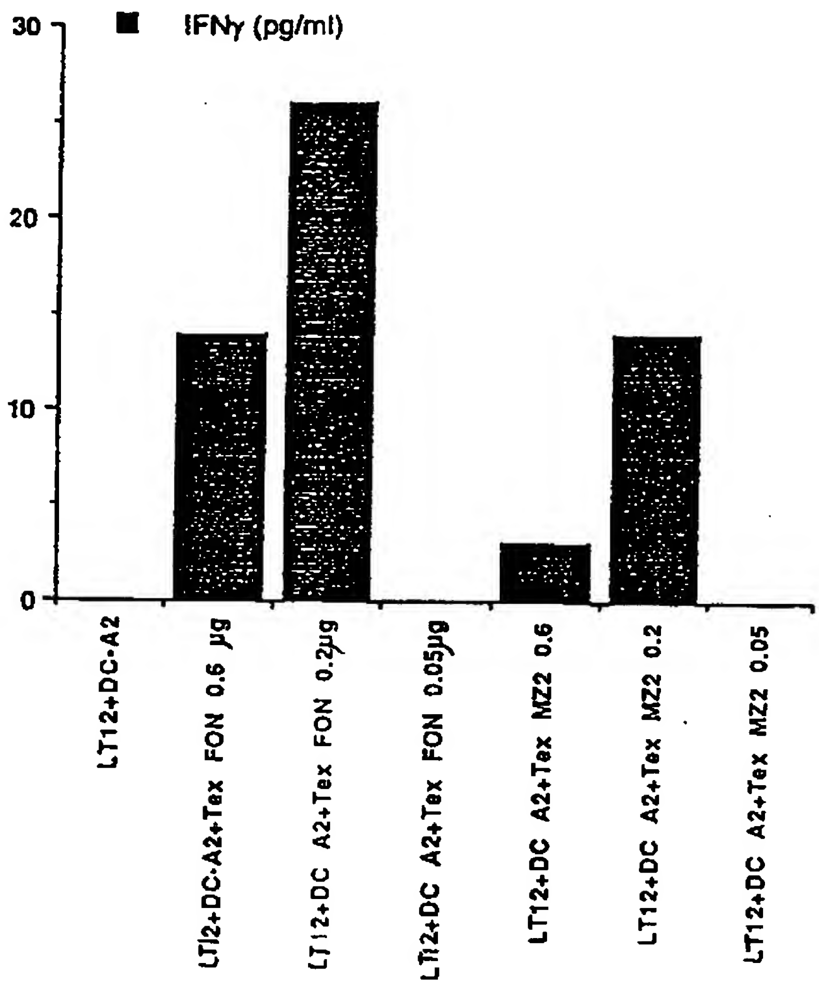
IFNγ (pg/ml)
30
20
10
0
LT12+DC-A2
LT12+DC-A2+Tex FON 0.6 µg
LT12+DC A2+Tex FON 0.2µg
LT12+DC A2+Tex FON 0.05µg
LT12+DC A2+Tex MZ2 0.6
LT12+DC A2+Tex MZ2 0.2
LT12+DC A2+Tex MZ2 0.05

Figure 5.

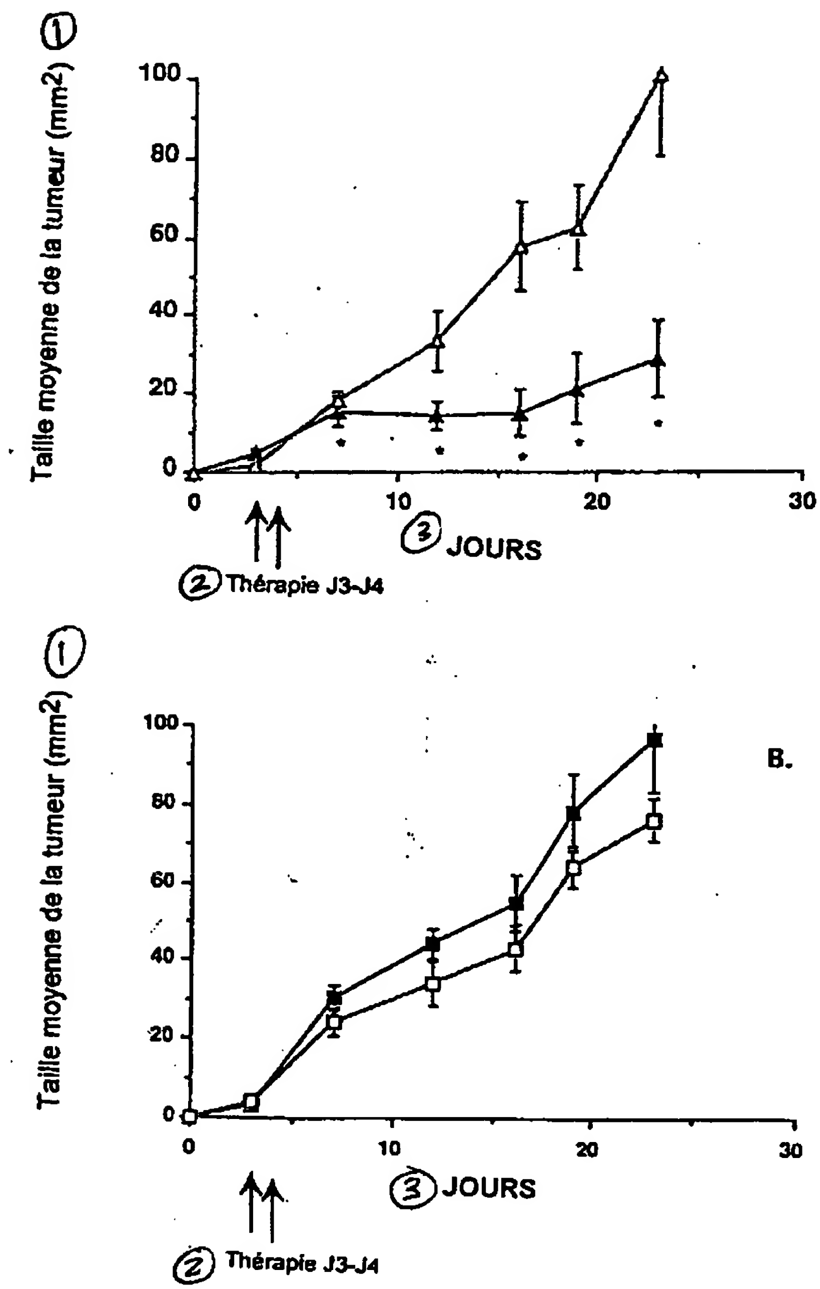

Figure 6.

[Key: 1. Average tumor size; 2. Therapy Day 3 - Day 4; 3. Days]

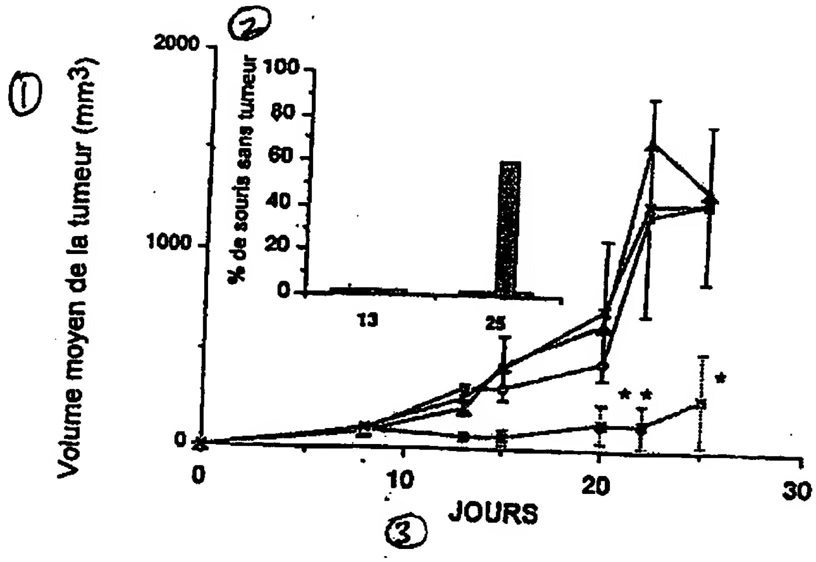

Figure 7.

[Key: 1. Average tumor size; 2. % of tumor-free mice; 3. Days]

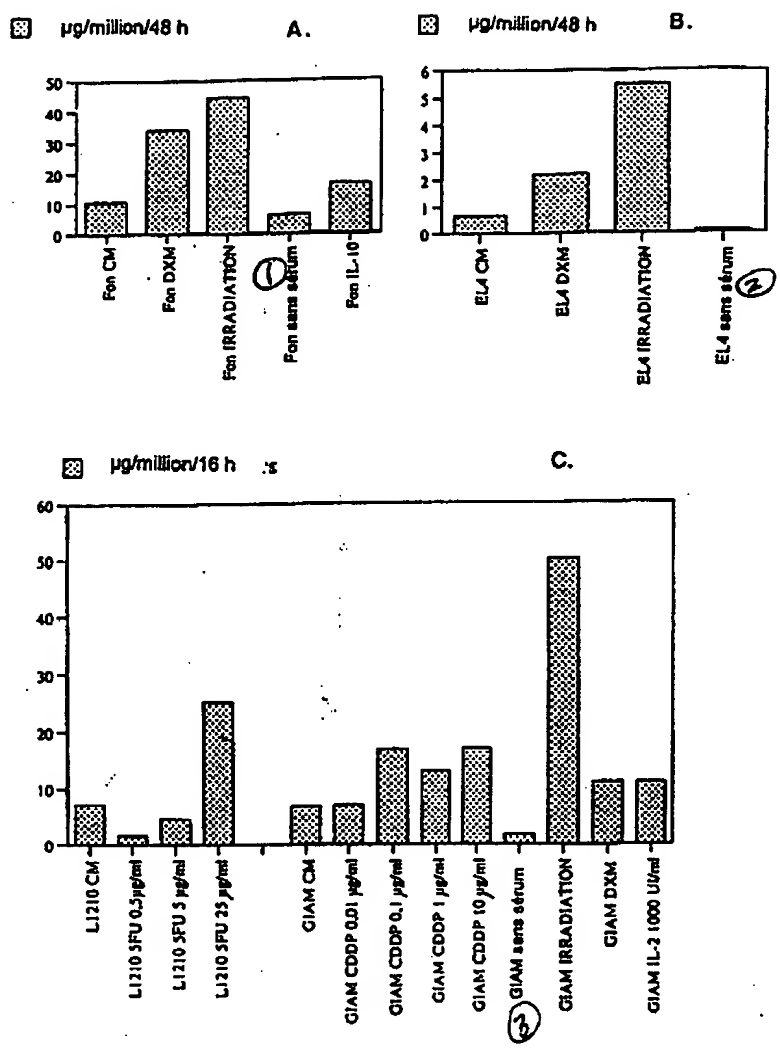

Figure 8.

[Key: 1. FON without serum; 2. EL4 without serum; 3. GIAM without serum]

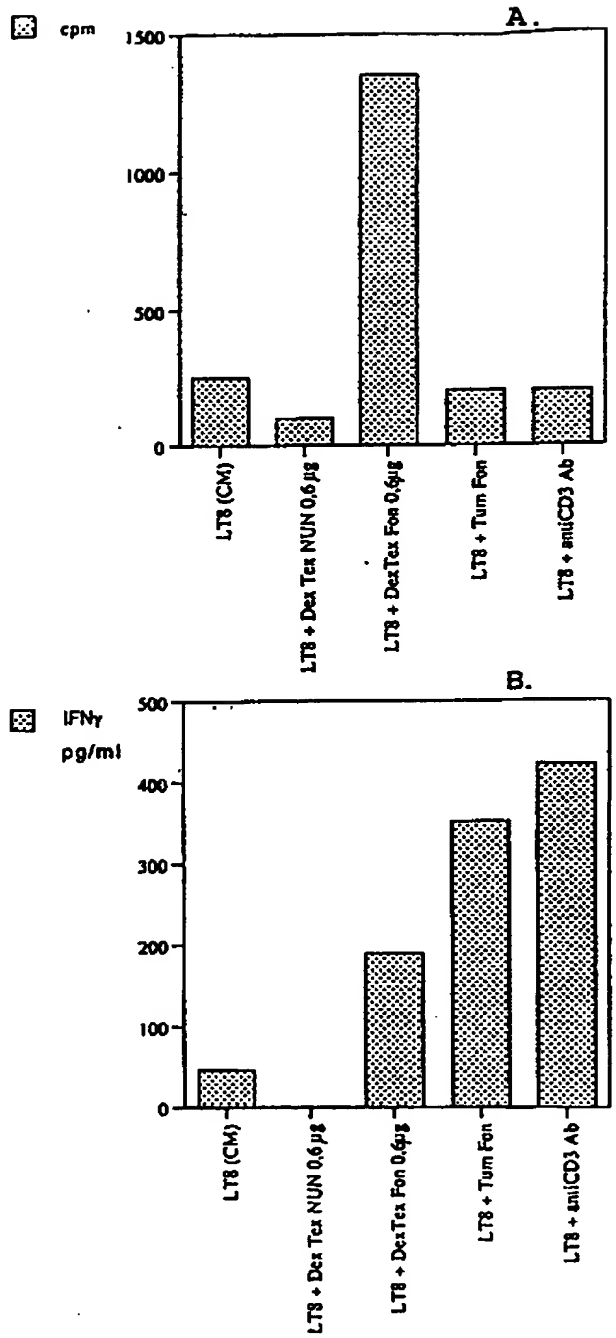
A.
cpm
1500
1000
500
0
LT8 (CM)
LT8 + Dex Tex NUN 0,6 µg
LT8 + DexTex Fon 0,6µg
LT8 + Tum Fon
LT8 + antiCD3 Ab
B.
IFNγ pg/ml
500
400
300
200
100
0
LT8 (CM)
LT8 + Dex Tex NUN 0,6 µg
LT8 + DexTex Fon 0,6µg
LT8 + Tum Fon
LT8 + antiCD3 Ab

Figure 9.

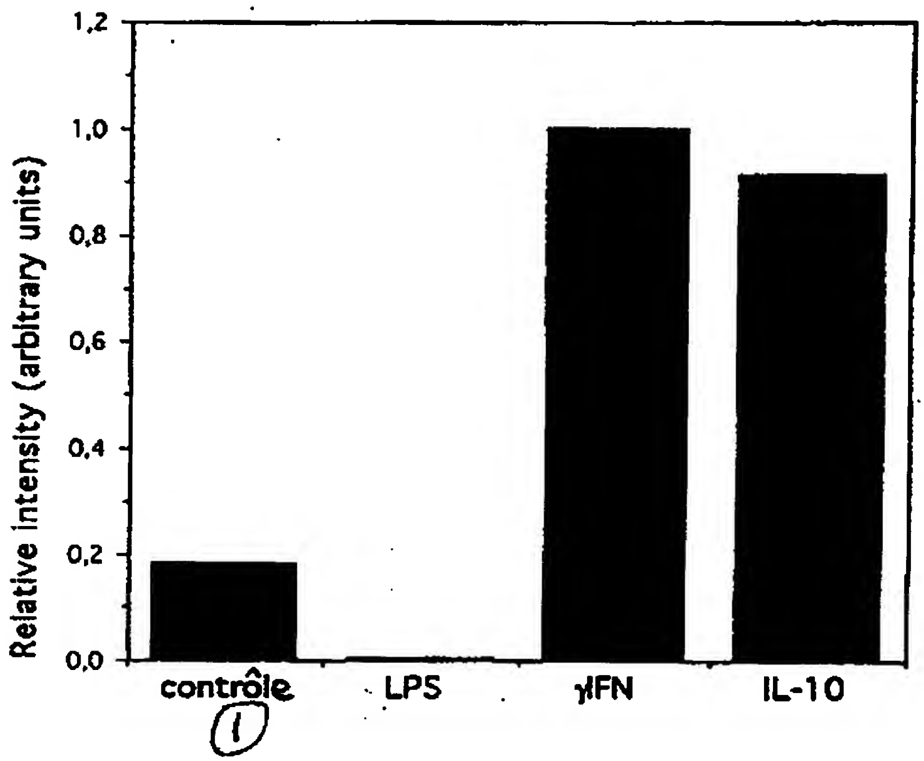

Figure 9C.

[Key: 1. Control]

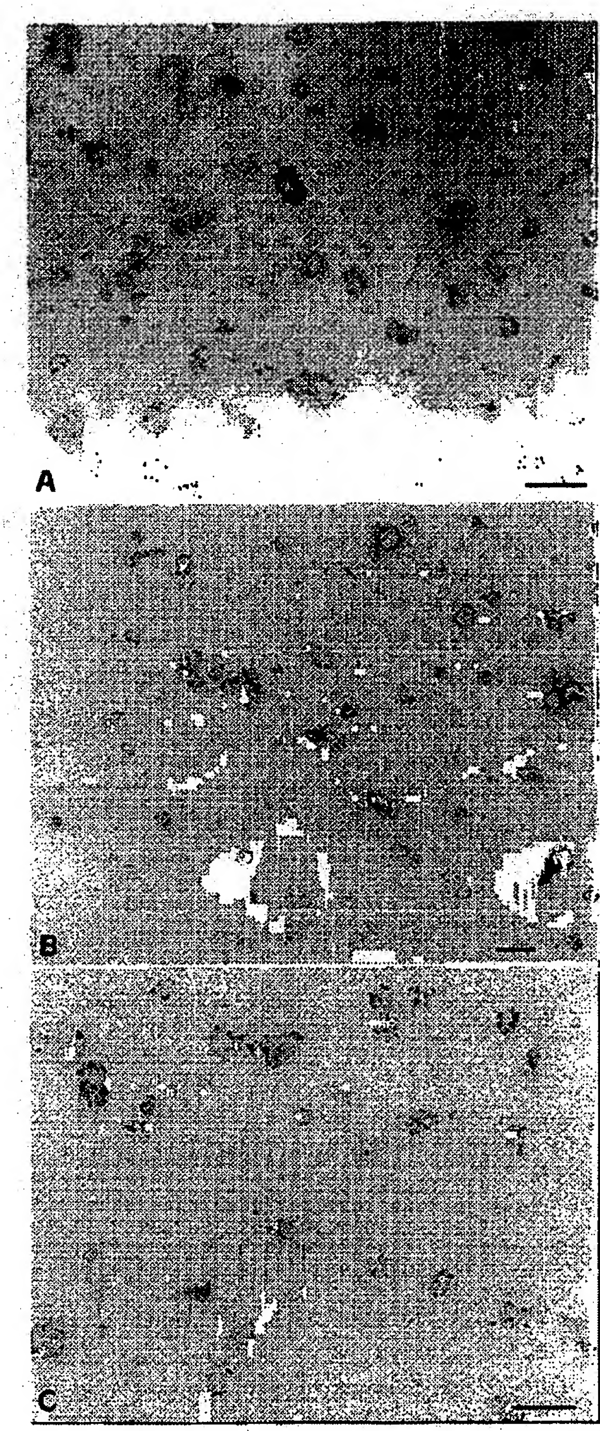

Figure 10.

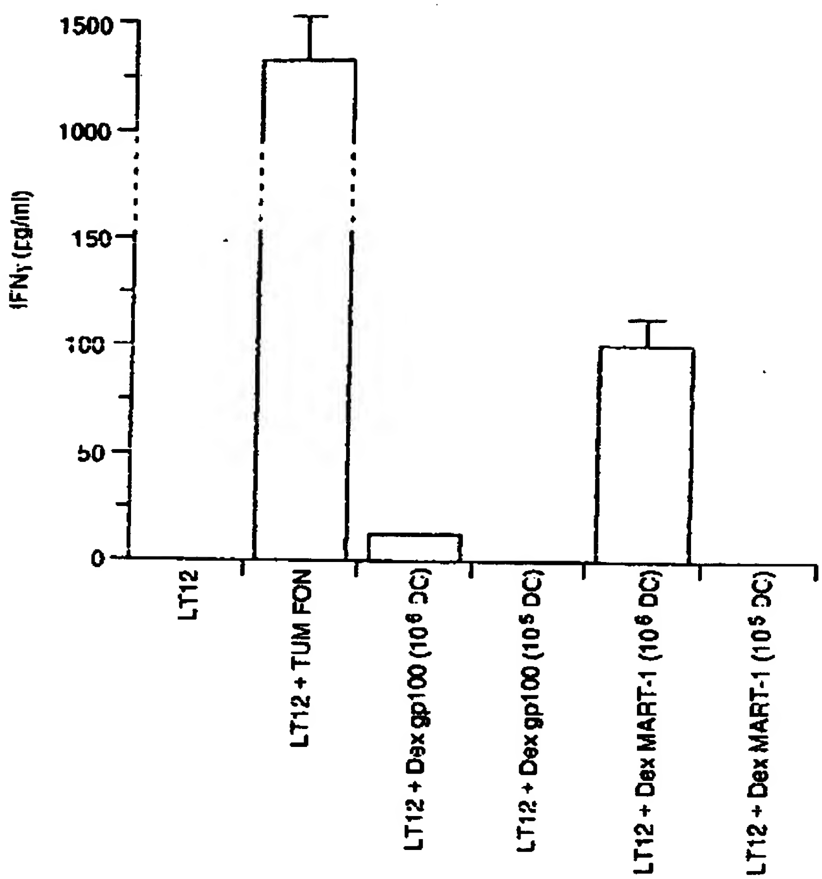
1500
1000
150
100
50
0
IFNγ (pg/ml)
LT12
LT12 + TUM FON
LT12 + Dex gp100 (10⁶ DC)
LT12 + Dex gp100 (10⁵ DC)
LT12 + Dex MART-1 (10⁶ DC)
LT12 + Dex MART-1 (10⁵ DC)

Figure 11.

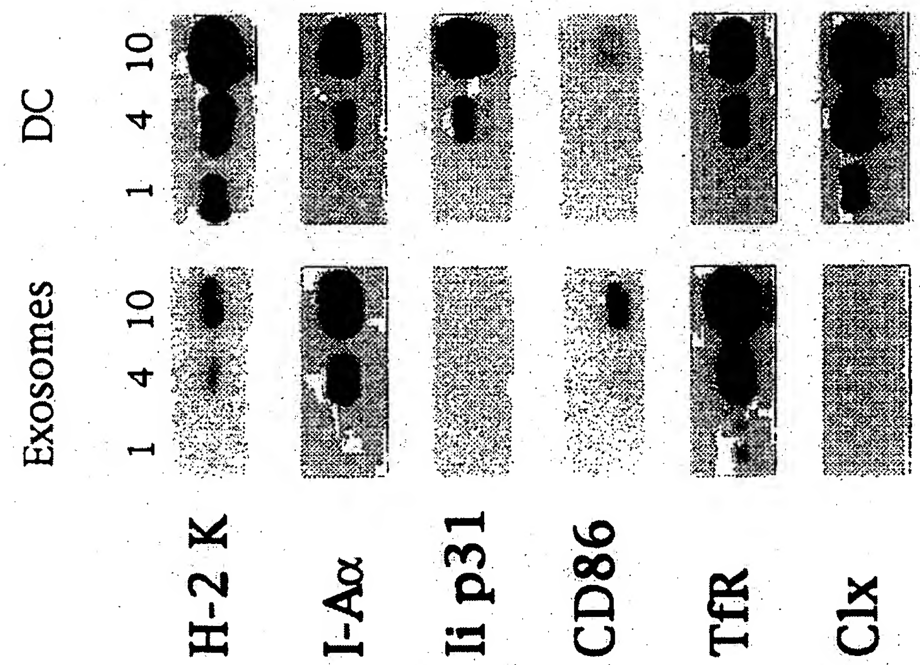

Figure 12.

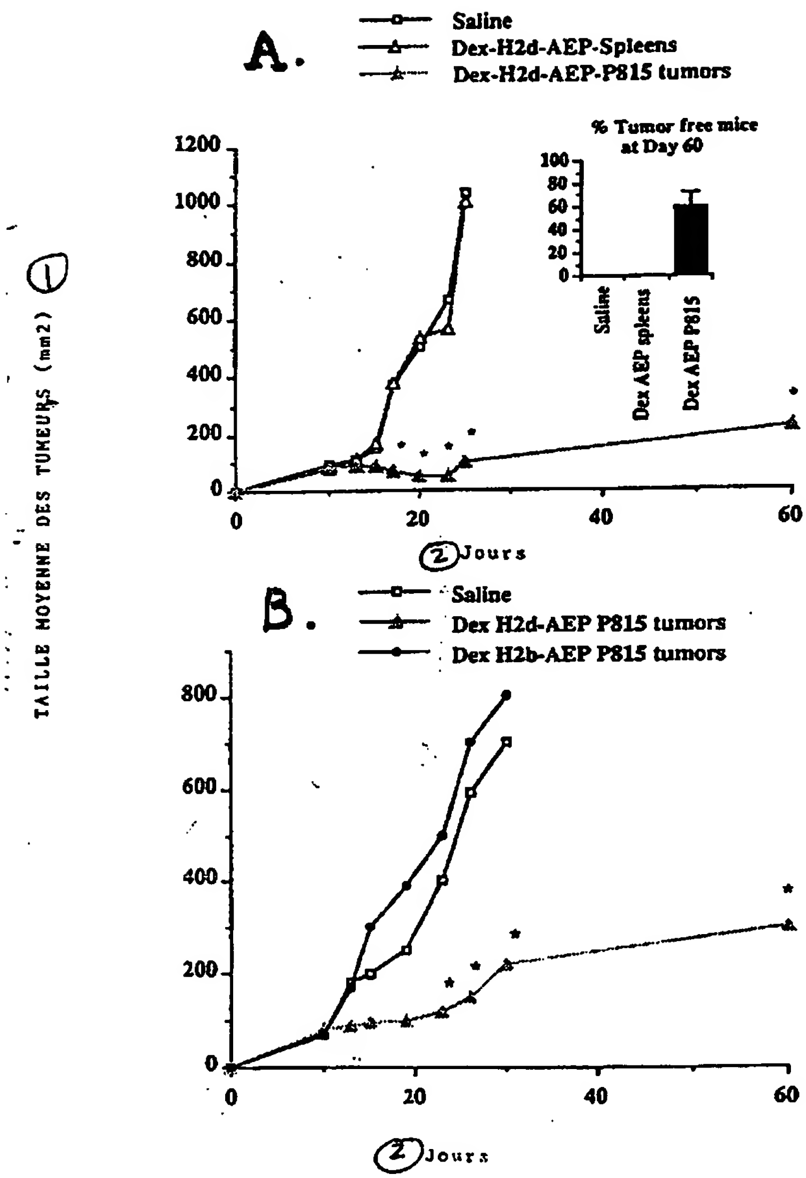

Figure 13.

[Key: 1. Average tumor size; 2. Days]

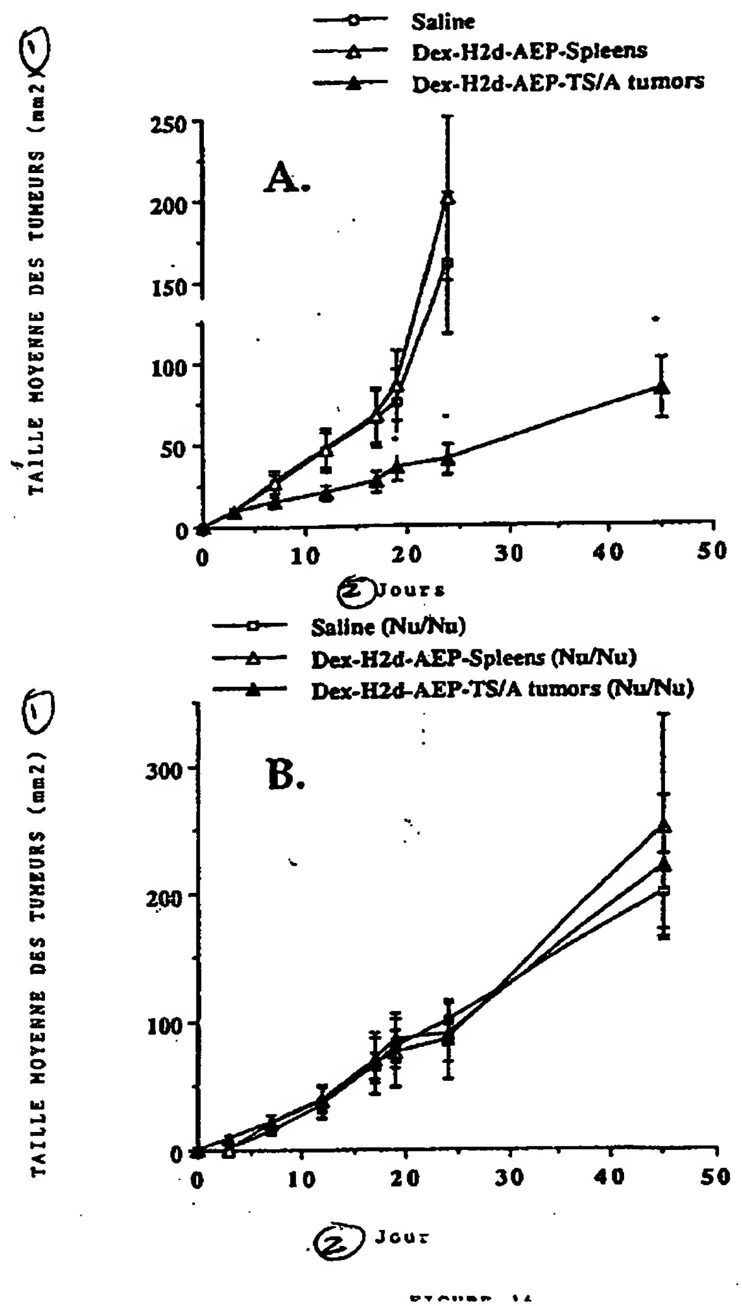

Figure 14.

[Key: 1. Average tumor size; 2. Days]

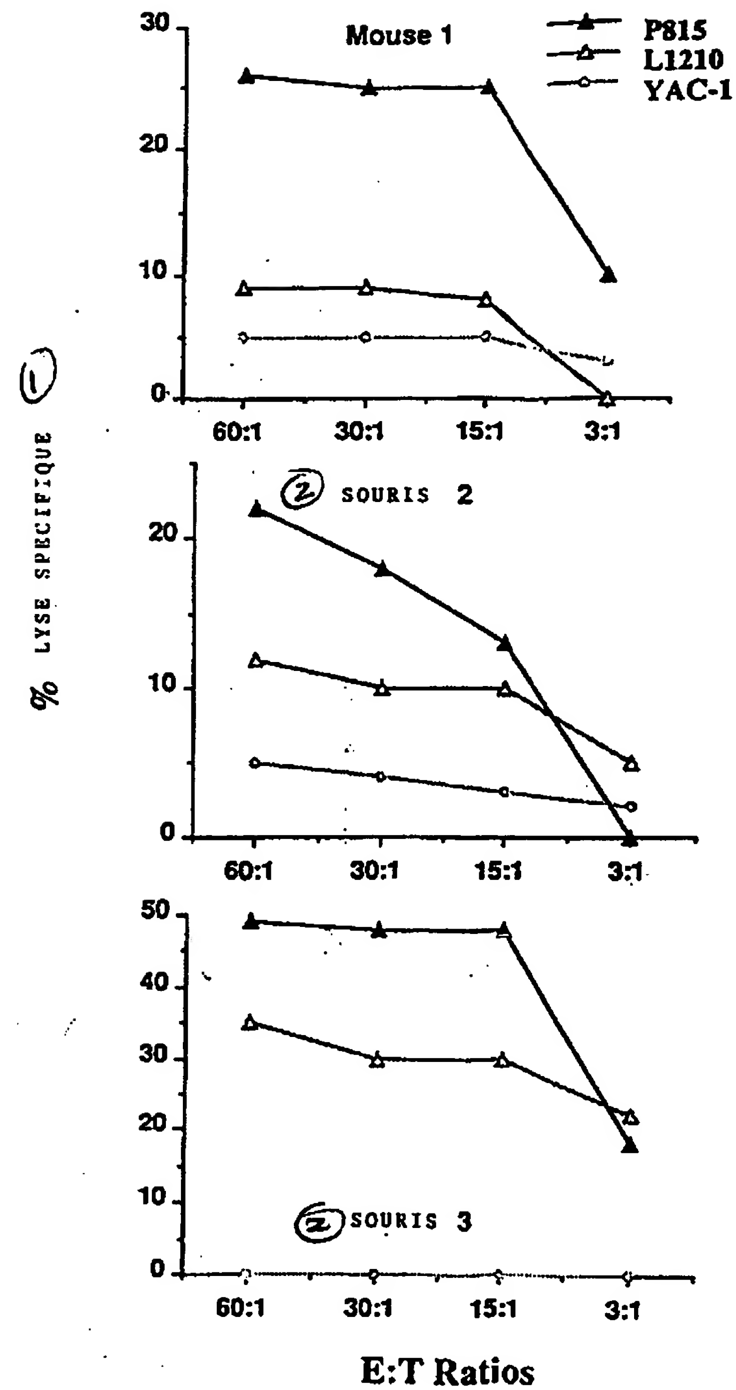

Figure 15.

[Key: 1. % Specific lysis; 2. Mouse]

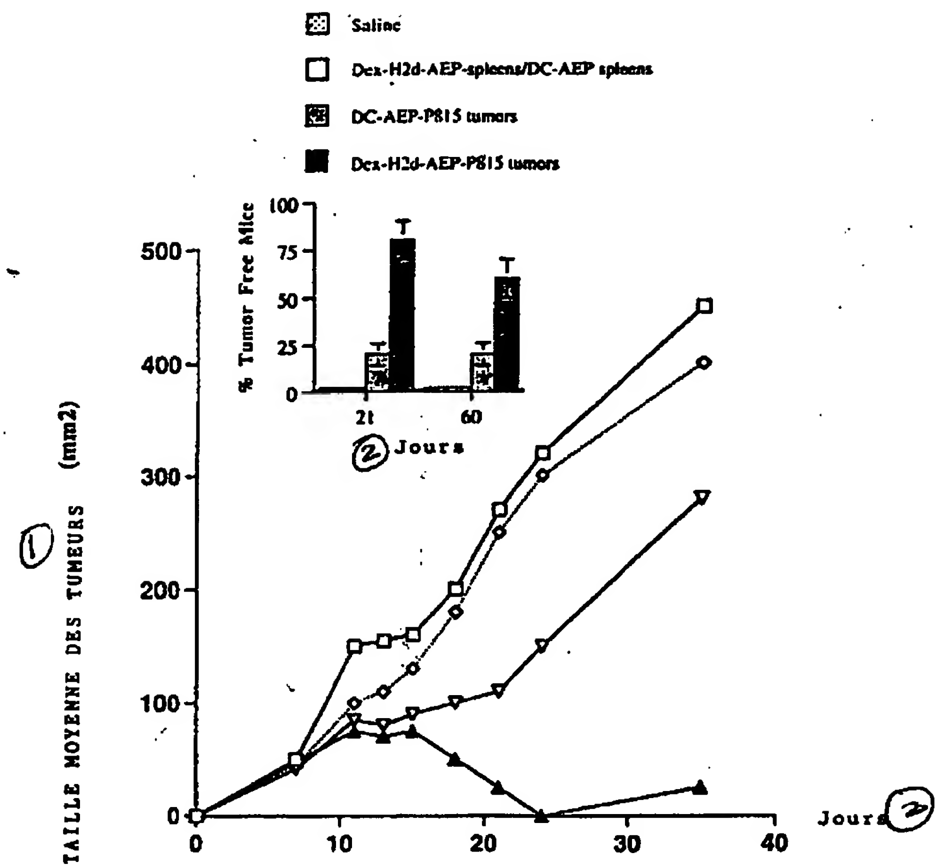

Figure 16.

[Key: 1 Average tumor size; 2. Days]

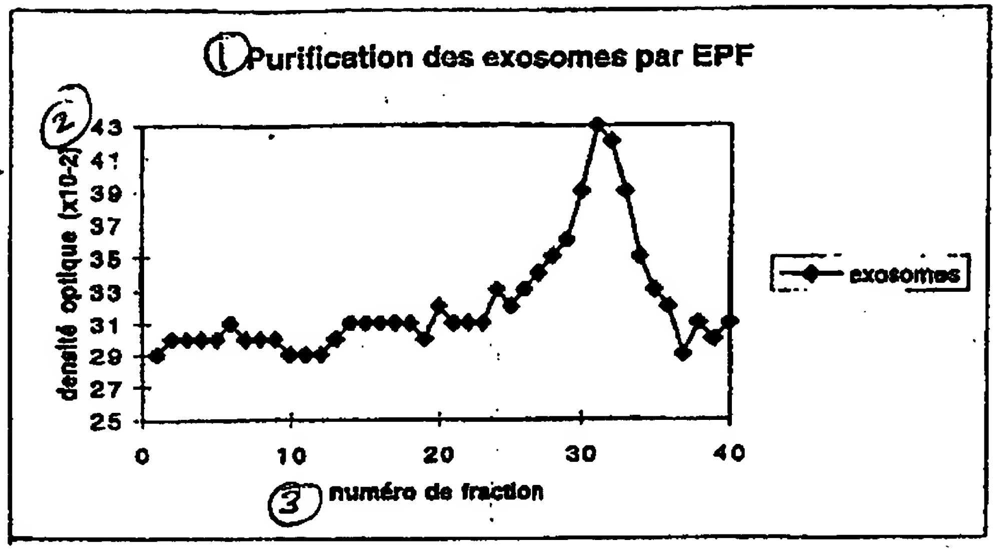

Figure 17.

[Key: Exosome purification by electrophoresis in fluid phase; 2. Optical density; 3. Fraction number]